Université de Montréal

# Comparison of proteins of the endoplasmic reticulum from control rat liver with proteins of the endoplasmic reticulum from dissected liver tumor nodules

par

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Ce mémoire intitulé:

**Comparison of proteins of the endoplasmic reticulum from control rat liver with proteins of the endoplasmic reticulum from dissected liver tumor nodules**

Présentée par:
Eman Abdou

A été évalué par un jury composé des personnes suivantes:

Docteur Dorin-Lucian Ghitescu.....président-rapporteur
Docteur Jacques Paiement.....directeur de recherche
Docteur Nicole Leclerc.....membre du jury

# DEDICATION

***TO MY BELOVED GRANDPARENTS, PARENTS, MY SISTER AND BROTHER.***

***TO MY DEAR AUNT AND UNCLES AND TO MY COUSINS.***

*SPECIAL DEDICATION TO MY BELOVED:*
***SALMA***

# SOMMAIRE

Le carcinome hépatocellulaire (CHC) est parmi les tumeurs malignes le plus répandu au monde (Parkin et al., 2005). Une intoxication à l'aflatoxine (une hépatotoxine puissante) est parmi les causes majeures de cancer de foie dans le monde (Bennett and Klich, 2003; Carnaghan, 1964).

On a comparé la composition protéique du réticulum endoplasmique (RE) de foie de rats témoins avec la composition protéique du RE de nodules tumoraux disséqués de foie de rats traités avec l'aflatoxine B1. La spectrométrie de masse, le compte de peptide et la validation par immunobuvardage ont été utilisés pour identifier et pour déterminer l'expression relative de protéines dans les fractions enrichies de RE.

Le RE est une organelle clé pour la voie de sécrétion. Il est impliqué dans la biosynthèse des protéines et des lipides (Shibata et al., 2006). Aussi, il est impliqué dans la dégradation des protéines et la détoxication des produits toxiques (Lavoie and Paiement, 2008). Ces divers fonctions sont effectuées par de nombreuses protéines certaines sont identifiées dans ce mémoire et elles sont comparées avec les mêmes protéines dans le cancer.

Beaucoup de protéines liées au cancer étaient surexprimées dans le RE dérivé des nodules tumoraux. Parmi les protéines surexprimées on note l'annexine II, les produits des gènes activés par l'aflatoxine B1 (exemple GST pi), les inhibiteurs de l'apoptose (exemples tripeptidyl peptidase II et nucleophosmine), des protéines impliquées dans la dégradation protéique ubiquitine-dépendante (sous-unités de protéasome), des protéines impliquées dans le métabolisme de l'ARN méssager (exemples hnRNP K, hnRNP M et PABP 1), des protéines impliquées dans la transcription (exemple Y box protéine 1), des protéines impliquées dans la traduction (exemples eIF 2α et eEF 2) et des protéines des filaments intermédiaires du cytosquelette (exemples vimentine et cytokératine 19).

Plusieurs protéines impliquées dans la détoxication (exemple, protéines de la famille du cytochrome p450) étaient sousexprimées dans le RE de tumeurs. D'autres protéines impliquées dans la détoxication étaient surexprimées (protéines de la famille des aldo-keto réductases).

Au contraire, les protéines chaperones (exemples, Bip et calreticuline) et les protéines du complexe MHC class I qui est impliqué dans la présentation antigénique étaient en quantité similaire dans le RE de tumeur et dans le RE de foie témoin. Des protéines phosphorylées à un résidu tyrosine incluant ATP citrate lyase qui est le substrat de la voie de signalisation Akt, étaient présentes en quantité significative dans le RE de tumeur.

Les études d'immunobuvardage employant dix-huit anticorps différents ont confirmés la distribution relative de protéines entre le RE de tumeurs et celui de foie contrôle. Donc ces résultats ont permit de valider les résultats obtenu par spectrométrie de masse.

En conclusion, la composition protéique du RE des hépatocytes tumorales est différente de celle du RE de hépatocytes normaux. Cette différence favorise probablement la survie des cellules cancérologique du foie. En plus, plusieurs protéines inconnues étaient présentes en concentration plus grande dans le RE de tumeur comparé à celle dans le RE de foie de rats témoins. Certaines de ces protéines inconnues pourraient être devenir de nouveaux marqueurs de tumeurs.

**Mots clés:** réticulum endoplasmique, carcinome hépatocellulaire, immunobuvardage, protéomique, spectrométrie de masse, protéines phosphorylées, protéines inconnues, biomarqueurs.

# ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (Parkin et al., 2005). Aflatoxin (a potent hepatoxin) is one of the major causes of liver cancer in the world (Bennett and Klich, 2003; Carnaghan, 1964).

We have compared the protein composition of endoplasmic reticulum (ER) from control rat liver with the composition of ER from dissected liver tumor nodules from rats treated with aflatoxin B1. Mass spectrometry, peptide counts and immunoblot validation were used to identify and determine the relative expression level of the proteins in highly enriched ER fractions

Normally, the ER is a key organelle of the secretion pathway involved in the biosynthesis of both proteins and lipids (Shibata et al., 2006). Also, it is involved in protein degradation and toxic products detoxification. These diverse functions of the ER are carried out via multiple proteins (Lavoie and Paiement, 2008).

Many proteins relevant to cancer were overexpressed in ER from dissected liver tumor nodules. These include examples as Annexin II, the classical aflatoxin B1 gene targets (i.e. GST pi), inhibitors of apoptosis (i.e. tripeptidyl peptidase II and nucleophosmin), proteins involved in proteasome metabolism (i.e. proteasome subunits), proteins involved in mRNA metabolism (i.e. hnRNP K, hnRNP M and PABP 1), in transcription (i.e. Y box protein 1) in translation (i.e. eIF 2α and eEF 2) and intermediate filaments cytoskeleton proteins (i.e. vimentin and cytokertain 19). Many proteins implicated in the detoxification (examples include the cytochrome p450 proteins) were underexpressed whereas a number of aldo-keto reducatases proteins were overexpressed in ER from tumors. In contrast, proteins of the folding machinery (i.e. Bip and calreticulin) and proteins of the MHC class I peptide loading complex were almost expressed in equal amounts in ER from dissected tumor nodules compared to those in

control ER. Tyrosine phosphorylated proteins including ATP citrate lyase a substrate of the Akt signaling pathway were observed in a significant quantity in cancer ER preparations. Many proteins of unknown function were observed in either concentration in hepatic cancer ER membranes.

Eighteen antibodies confirmed the relative distribution of immunoblotted proteins. These different antibodies confirmed the relative distribution of proteins of ER from tumor and control liver. Therefore, these results allowed the validation of the results obtained by mass spectrometry.

Therefore, in conclusion the proteomic composition of the ER of cells in HCC is different from those of ER of normal hepatocytes. This difference is probably an advantage for the survival of tumoral cells in liver cancer. Furthermore, many known proteins that were observed in greater concentration in cancer ER as compared to the ER from control liver, can be as potential tumor markers in HCC (examples include eEF2, GST pi and vimentin).

**Key words:** endoplasmic reticulum, hepatocellular carcinoma, immunoblot, proteomics, mass spectrometry, phosphorylated proteins, unknown proteins, biomarkers.

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| | |
|---|---|
| AFB1 | Aflatoxin B1 |
| rAFAR 2 | Rat aflatoxin B1 aldehyde reductase member 2 |
| rAFAR 1 | Rat aflatoxin B1 aldehyde reductase member 1 |
| AFP | Alpha fetoprotein |
| AKRs | Aldo-keto reductases |
| ATP | Adenosine triphosphate |
| BiP/GRP 78 | Immunoglobulin heavy chain-binding protein/78 kDa glucose-regulated protein precursor |
| CYP 450 | Cytochrome p450 family protein |
| DNA | Deoxyribonucleic acid |
| eIF 2α | Eukaryotic translation initiation factor 2, subunit 1 alpha |
| eEF 2 | Eukaryotic translation elongation factor 2 |
| ER | Endoplasmic reticulum |
| GAS 2 | Growth arrest-specific protein 2 |
| GST | Glutathione S-transferase |
| hnRNP K | Heterogeneous nuclear ribonucleoprotein complex K |
| LRP/MVP | Lung resistance related protein/major vault protein |
| MHC | Major histocompatibility complex |
| MS | Mass spectrometry |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NERA | Novel endoplasmic reticulum associated protein |
| PABP 1 | Polyadenylate-binding protein 1/Poly(A)-binding protein 1 |
| PA2G4/EBP 1 | Proliferation-associated protein 2G4/ErbB3 binding protein 1 |
| PAGE | Polyacrylamide gel electrophoresis |
| PDI | Protein disulfide isomerase |
| PLC | Protein loading complex |
| RER | Rough Endoplasmic reticulum |
| Ribo S6 | Ribosomal protein S6 |

| | |
|---|---|
| RNA | Ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| RM | Rough microsomes |
| RMNod/rmnod | Rough microsomes from dissected liver tumor nodules |
| S100Ctl | From cytosolic fraction from control rat liver |
| S100Nod | From cytosolic fraction from dissected liver tumor nodules |
| SER | Smooth endoplasmic reticulum |
| SMCtl/smctl | Smooth microsomes from control rat liver |
| SMNod/smnod | Smooth microsomes from dissected liver tumor nodules |
| tER | Transitional ER |
| VCP | Valosin-containing protein |
| YB 1 | Y box protein 1 |

# INTRODUCTION

## 1 Liver cell tumor: Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma is the most common primary malignant disease of the liver (Kew, 2002). It signifies a tumor arising from the epithelial layer of the liver (Suriawinata and Thung, 2002).

### 1.1 Epidemiology

Hepatocellular carcinoma is the sixth most common cancer worldwide and is the third leading cause of cancer-related deaths worldwide (Parkin et al., 2005). Although the incidence of HCC increases with age, the age of peak incidence vary with population (Suriawinata and Thung, 2002). For example, it occurs at earlier ages in black African and ethnic Chinese populations. Men are more affected than women. Male to female ratio is 3:1 in high incidence regions (Kew, 2002) and a lower ratio is found in low-incidence regions (Parkin et al., 2005). The highest incidences are found to be in Asia (eastern and southeastern), some of the sub-Saharan Africa and Melanesia and the lowest are found to be in developed areas (except for southern Europe), Latin America, and south-central Asia (Parkin et al., 2005).

### 1.2 Etiological factors

Hepatocellular carcinoma is associated with chronic liver disease, mainly cirrhosis (Borzio et al., 1998; Motola-Kuba et al., 2006; Thorgeirsson and Grisham, 2002). Cirrhosis of the liver may derive from a variety of factors including, chronic hepatitis viral infections (hepatitis B virus and hepatitis C virus), alcoholism, AFB1 intoxication and genetic diseases such as hemochromatosis (Mazzanti et al., 2008), Wilson's disease, alpha-1 antiprotease (antitrypsin) deficiency (Fattovich et al., 2004). Hence, cirrhosis can be considered as a premalignant condition (Fattovich et al., 2004).

## 1.3 Clinical picture

Clinical presentations can be right upper quadrant abdominal pain (where is the anatomic location of the liver organ), early satiety, and weight loss. Others can include spontaneous rupture of the tumor into the peritoneal cavity, obstructive jaundice, or bony pain from metastasis. Moreover, paraneoplastic syndromes may occur leading to erythrocytosis (due to production of erythropoietin), hypoglycaemia (due to production of an insulin-like growth factor), and hypercalcemia (due to production of parathyroid-related protein). Physical findings may be enlarged liver upon palpation and a vascular bruit on auscultation, consistent with hypervascularity of the tumor (Parikh and Hyman, 2007).

## 1.4 Diagnosis

Although there is no definite evidence that screening in liver cancer improves survival, many hepatologists screen patients in high-risk groups with either serum alpha-fetoprotein and/or ultrasound of the liver (Parikh and Hyman, 2007). The biopsy of the tumor is an important tool to establish diagnosis as well. However, the considerable false negative rate from biopsy of lesions less than 2 cm makes a negative biopsy does not conclusively rule out the diagnosis of HCC. Also, among the most important downsides of the invasive biopsy of liver tumor includes risk of tumor seeding (1%-2%) and its limitation by general contraindications (ascites, decreased coagulation factors, thrombocytopenia) due to a risk of bleeding (Durand et al., 2007; Parikh and Hyman, 2007). A recommendation stated that an ultrasound of the liver every 6 months in high-risk patients to screen for hepatocellular cancer would be helpful (Parikh and Hyman, 2007). Hence, markers are needed to correctly diagnose HCC (Yoon et al., 2006).

On the other hand, many staging and scoring systems are available for clinicians in their routine practice to determine the stage of the disease of their patients. Pons and colleagues (Pons et al., 2005) grouped these different tools in a relatively recent article. Examples of these tools include the Okuda and Tumor-Lymph Node-Metastasis (TNM) staging systems. The latter is the most commonly used staging system for solid tumors in

general, but has severe limitations because it does not put in consideration the severity of underlying cirrhosis (Parikh and Hyman, 2007).

Genetic analysis is thought to be a way to classify tumors. Since then there have been several studies of genetic analysis of HCC (Wong et al., 1999). Nevertheless, such genetic studies should be validated using other techniques examples include immunohistochemistry and immunoblot.

Finally, another emerging method of classification of HCC can be according to phenotypic (differentiation) markers. For that, immunohistochemistry was used to study the expression of different differentiation markers that included hepatocytic differentiation marker (HEP-PAR-reactive antigen), biliary differentiation markers (AEl-AE3, cytokeratin-19), proliferation markers (Ki-67, proliferating cell nuclear antigen), AFP, p53, and transforming growth factor alpha in the tumor tissue (Wu et al., 1996).

**1.5 Prevention and treatment**

Prevention of cancer can be achieved by preventing the development of cirrhosis and proper management cirrhosis (Fattovich et al., 2004) in order to preserve the liver function. This can be achieved by the use of a vaccine against hepatitis B virus (Chang et al., 2005), treatment of chronic hepatitis C infection by combination of interferon alpha and ribavirin (Patel et al., 2006), early detection of inherited liver diseases as well as better management of alcohol intake, examples include encouraging persons in alcoholics anonymous participation (Mann et al., 2005) and prevention of aflatoxin intoxication, examples include applying post-harvesting technologies to limit fungus growth and crop contamination (Lodato et al., 2006).

Treatment can be divided into four categories: surgical interventions (tumor resection and liver transplantation), percutaneous interventions (ethanol injection, radiofrequency thermal ablation), transarterial interventions (embolization, chemoperfusion, or chemoembolization) and drugs including gene and immune therapy. Curative treatment that can result in complete response and improved survival in many

cases can be tumor resection, liver transplantation, and percutaneous interventions. On the other hand, palliative treatment that can improve survival and patient's quality of life in some cases may include transarterial interventions. Finally, drugs and conventional radiotherapy have no proven efficacy (Blum, 2005). However, in the long-term management for HCC patients the only definitive treatment option remains to be liver transplantation (Krige and Beckingham, 2001a; Krige and Beckingham, 2001b; Llovet et al., 2003; Parikh and Hyman, 2007). This is because in case of liver transplantation both the tumor and the liver dysfunction due to the underlying cancer-prone cirrhotic liver are definitively cured (Parikh and Hyman, 2007). However, this therapeutic approach has limitations because of the shortage of organ donations (Parikh and Hyman, 2007).

### 1.6 Survival

Survival rates are 3% - 5% in United States and developing countries (Parkin et al., 2005). In patients with preserved liver function and without portal hypertension, the five-year survival rate is more than 70 % after surgical resection of the tumor (Bruix et al., 1996; Mazzanti et al., 2008). On the other hand, patients with advanced liver disease, liver transplantation offers one year and five-year survival rates of 80% and 60% respectively (Krige and Beckingham, 2001a).

## 2 Aflatoxin B1 (AFB1)

In 1967, Halver (Halver, 1967) mentioned the early role played by rainbow trout (a species from the salmon family) in the discovery of the carcinogenic action of AFB1. AFB1 is now a well-known mycotoxin that affects largely the crop product (such as corn and rice) especially if improperly stored. It is produced by certain strains of fungi namely; Aspergillus flavus, Penicillium, Fusiarium, Claviceps and Stachybotrys (Meissonnier et al., 2007; Smela et al., 2001). It is principally produced by Aspergillus flavus and Aspergillus parasiticus which are common in most soils and are usually involved in decay of plant materials (Grishin, 2005).

Mycotoxicoses would describe the group of disease that develops secondary to consumption of mycotoxins (Kiessling, 1986). The mechanism of mycotoxicity involves interference with various aspects of cell metabolism. The toxins vary in specificity and potency for their target cells, cell structures or cell processes by species and strain that produces them, producing neurotoxic, teratogenic or carcinogenic effects. The latter effect is the one of our interest here in this work. Aflatoxins B1, B2, G1, and G2 are produced in grains in both field and storage. The four compounds are distinguished based on their fluorescence colour under the effect of the long-wave ultraviolet illumination (hence B is for blue and G is for green), with the subscripts relating to their relative chromatographic mobility. AFB1 is usually found in the highest concentrations, followed by AFG1, AFB2, and AFG2. The toxins pose a significant public health concern as diseases resulting from ingestion of aflatoxins, include acute liver disease to cancer development (McLean and Dutton, 1995).

**2.1 Aflatoxin B1 (AFB1) metabolism**

AFB1 is a specifically metabolized by the action of the mixed function mono-oxygenase enzyme systems (cytochrome P450-dependent) in the tissues (particularly the liver) of the affected animal into 8,9-epoxide form (activated and mutagenic form) and others (detoxication products) (McLean and Dutton, 1995). This epoxide form is highly reactive and interacts with several cellular macromolecules, such as DNA, RNA and protein (Grishin, 2005; McLean and Dutton, 1995). Consequently, AFB1 can affect the liver and can cause serious adverse effects, hence known to be the most potent hepatotoxin and hepatocarcinogen (Carnaghan, 1964).

Aldo-keto reductases (AKRs) are a superfamily of proteins that among its members are those that deal with aflatoxin detoxification: for example, AKR7A2/A3 known as Aflatoxin aldehyde reductases. It is noted that A2 member is widely distributed in human tissues while the A3 member is expressed in the liver, colon, kidney, and pancreas. These proteins can reduce the dialdehyde protein-binding form of aflatoxin B1 (AFB1) to the non-binding AFB1 dialcohol (Jin and Penning, 2007).

### 2.2 Aflatoxin B1 (AFB1) as a chemical carcinogen

AFB1 is considered one of the chemicals that can induce carcinogenesis, hence known as a chemical carcinogen. It enhances cancer development and progression. In fact, carcinogenesis is considered as a multistep process. This includes three distinct (morphological and molecular) steps as follows: initiation (occult), promotion (reversible) and progression (increasing aggressiveness of the tumour and malignant conversion) (Pitot, 2001) in which control of cell growth is progressively perturbed (Mazzanti et al., 2008). AFB1 is a potent mutagen (Kobertz et al., 1997). Exposure to AFB1 as the case in high contamination of cereal food was found to be in close association with development of specific mutant p53 gene that can consequently favor recurrent HCC. The carcinogenic potential of AFB1 vary between individuals, as for the same exposition not all patients will develop HCC (Laurent-Puig and Zucman-Rossi, 2006). Normally the p53 gene functions as a tumor suppressor (Hanahan and Weinberg, 2000) and is expressed at a low level in the cell. Its up-regulation (in response to various stimuli as DNA damage, hypoxia, viral proteins, or telomere erosion and oncogene activation) will end in the induction of pathways that lead to either cell cycle arrest or apoptosis (Vousden, 2000). One of the first measurable effects of AFB1, on cells and tissues is inhibition of DNA synthesis (McLean and Dutton, 1995). Practically speaking, any malignant growth is in need of six basic alteration in the cell physiology; namely self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastases (Hanahan and Weinberg, 2000).

## 3 Endoplasmic reticulum (ER)

The endoplasmic reticulum (ER) is widely present in all eukaryotic cells being an essential dynamic subcellular organelle for cell life. It plays an important role in the biosynthesis and modification of membrane and secretory proteins and the biosynthesis of lipids. It also plays a role in calcium homeostasis, and degradation of misfolded proteins (Baumann and Walz, 2001; Lavoie and Paiement, 2008; Shibata et al., 2006).

### 3.1 General structure of the ER

The ER membrane is organized into a network of branching tubules and flattened sacs extending throughout the cytosol (Shibata et al., 2006; Voeltz et al., 2006). The tubules and sacs are continuous and enclose a single internal space called the ER lumen (ER cisternal space) (Baumann and Walz, 2001).

### 3.2 Functions of ER

#### 3.2.1 Metabolism of proteins: Biosynthesis, transport and degradation

The ER is involved in the metabolism of proteins; this includes protein biosynthesis, transport and degradation. The biosynthesis of proteins takes place inside the ER (lumen). This is completed by the insurance of proper folding of newly synthesised protein (soluble proteins are folded within the lumen of the ER whereas membrane proteins are folded in three environments; the cytosol of the cell, the lipid bilayer of the ER and the ER lumen), correct disulfide bonding and posttranslational modifications (such as the addition of oligosaccharides). After that, the secretory pathway will ensure the transport of a given newly synthesised protein from ER to the Golgi apparatus (where in the latter, the protein will undergo further posttranslational modifications) then to its final destination at the cell surface. So like that, the newly synthesized proteins that fold, assemble and assume a stable conformation are recognized and sorted into secretory vesicles by the activity of the cytosolic coat protein complex COP I and II. The later are crucial for directing the sequential transfer of material between the ER and the Golgi complex. However, the newly synthesized proteins with unstable conformation are specifically targeted for degradation by an ER quality control process called ER associated degradation (ERAD). The ERAD will result in degradation of proteins by the cytosolic ubiquitin–proteasome system (Aridor, 2007; Brodsky and Fisher, 2008; Duden, 2003; Klumperman, 2000; Nickel et al., 1998; Pelham, 1996; Yorimitsu and Klionsky, 2007)

### 3.2.2 Metabolism of lipids: Biosynthesis and transport

The ER is also involved in the metabolism of lipids; this includes lipids' biosynthesis and transport. The lipogenic enzymes involved in the biosynthesis of lipids are bound to the cytosolic surface of the ER membrane. The secretory pathway will ensure the transport of a given newly synthesised lipid from ER to the Golgi apparatus then to its final destination as to the plasma membrane. In case of nascent cholesterol, it moves against a steep concentration gradient to reach the plasma membrane. Vesicular transport along the protein secretory pathway through the Golgi is one route from ER to plasma membrane (Baumann and Walz, 2001; Davis, 1999; Maxfield and Wustner, 2002; Nickel et al., 1998; Riezman and van Meer, 2004; Soccio and Breslow, 2004).

### 3.2.3 Metabolism of drugs

The ER is also involved in the metabolism of drugs; this also includes detoxification of xenobiotics by the detoxification machinery present in the ER, namely detoxification enzymes as cytochrome p450 family members (Cribb et al., 2005).

So disruption of the ER can lead to changes in protein synthesis and processing, dysregulation of lipid metabolism and accumulation of toxic compounds (Cribb et al., 2005). Therefore, the crucial metabolic role played by the ER made it a key organelle in development of many cellular pathological states, notably cancer events. Examples of other ER related diseases include cystic fibrosis, diabetes, neurodegenerative conditions and polycystic liver disease (reviewed in (Aridor, 2007). In addition the ER proteins are direct targets of hepatotoxic compounds (Cribb et al., 2005). Consequently, all the previously mentioned factors made the ER the most valid organelle target for our study.

### 3.3 Domains of the endoplasmic reticulum (ER)

The ER is composed of four main subdomains namely, rough ER (RER), smooth ER (SER), transitional ER (tER) and nuclear envelope (NE) (Lavoie and Paiement, 2008).

#### 3.3.1 Rough endoplasmic reticulum (RER)

The RER is the part of the ER where ribosomes are bound at its external surface hence given its name as rough (Palade, 1975). Normally, there are two forms of ribosomes in the cytosol. Membrane bound ribosomes (attached to the cytosolic side of the ER membrane), are engaged in the synthesis of proteins that are being concurrently translocated into the ER) and free unattached ribsomes involved in the synthesis of cytosolic proteins. Palade initially described these two forms of ribosomes (Palade, 1955). When a ribosome happens to be making a protein with a signal sequence, the signal directs the ribosome to the ER membrane via interaction with the signal recognition particle (Osborne et al., 2005).

#### 3.3.2 Smooth endoplasmic reticulum (SER)

It is the part of the ER, which is ribosome-free and hence is given its name as smooth (Shibata et al., 2006). In certain specialized cells (examples include liver cells, neurons and muscle cells), the SER is abundant and has additional functions (Voeltz et al., 2002). For example in case of liver cells, the SER contains the enzymes that are involved in the detoxification of various xenobiotics. Furthermore, there is an increase in the specific activity of cytochrome p450 family enzyme after treatment of the organism with phenobarbital (Kuriyama et al., 1969).

#### 3.3.3 Transitional endoplasmic reticulum (tER)

This represents the part of the ER showing areas confluent between the RER and the SER. The SER portion of the tER is often the origin of ER exit sites, which produce

transport vesicles carrying newly synthesized proteins and lipids to the Golgi apparatus for distribution in and out of the cell (Fan et al., 2003). Other names for this compartment include ER-Golgi intermediate compartment, vesiculo-tubular clusters or pre-Golgi intermediates (Hauri et al., 2000; Schweizer et al., 1988).

### 3.3.4 Nuclear envelope (NE)

This is a double membrane envelope, which is composed of an outer nuclear membrane, a nuclear pore, and an inner nuclear membrane. The nuclear pore complex (NPC) is anchored in the nuclear pore by transmembrane proteins that extend into the lumen of the nuclear envelope (NE). Moreover, the NPC acts as a doorkeeper by regulating the access of integral membrane proteins to the inner nuclear membrane (Hsia et al., 2007; Lusk et al., 2007). Thus, the NPC mediates the selective exchange of macromolecules between the nucleus and cytoplasm (Melcak et al., 2007). Figure 1 shows that the outer membrane of the NE is in continuity with RER and that both of them are covered with ribsosomes.

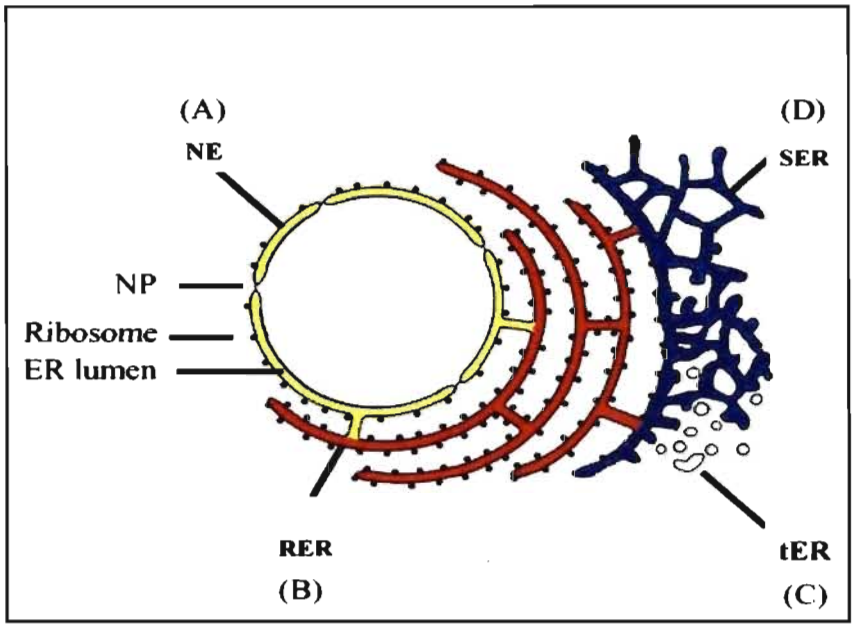


**FIGURE 1: Diagram of the four subcompartments of the endoplasmic reticulum.** (**A**) The nuclear envelope (NE) is shown with nuclear pores (NPs) and ribosomal particles attached to the outer membrane. (**B**) The rough ER (RER) is continuous with the NE and consists of stacked fattened saccules, whose limiting membranes have numerous attached ribosomal particles. (**C**) Transitional ER (tER) is composed of a RER subdomain continuous with a smooth ER (SER) subdomain consisting of buds and tubules devoid of associated ribosomes. (**D**) The smooth ER (SER) is devoid or almost free of ribosomal attachment and composed of a network of interconnecting tubules. The cisternal space forming the ER lumen is also shown (Modified from Lavoie and Paiement, 2008).

# 4 Proteomics

Proteomics is the study of the complete protein complement or the proteome of the cell. The proteome is dynamic and is in constant flux depending on the physiological state of the cell or depending on its pathological state (Cui et al., 2004). Hence, proteomic study allows the identification of protein changes caused by the disease. Protein changes occurring during neoplastic transformation affect cellular function and changes may include altered expression, differential protein modification, changes in specific activity, and unusual localization (Ai et al., 2006). Proteomics is currently considered as a powerful tool for studying protein expression and has been widely applied in field of cancer research (oncoproteomics) (Kolch et al., 2005; Sun et al., 2007). In addition, proteomics analysis can provide a profile of quantitative protein expression; this requires methods that are able to provide efficiently accurate and reproducible differential expression values for proteins in two or more biological samples (Kolkman et al., 2005). Proteomics involves the analysis of protein mixtures in a cellular environment (Drahos et al., 2005) or in an organelle (Brunet et al., 2003).

## 4.1 Organelle Proteomics

Organelle proteomics involve the study of proteins of intracellular organelles. This can be achieved initially by subcellular fractionation, which is the essential step among enrichment techniques in proteomics research, especially for study of organelles. Thus, subcellular fractionation allows the separation of organelles based on their physical or biological properties. In fact, subcellular fractionation is based on two major steps, which include homogenization (disruption of the cellular organization) and fractionation of the homogenate to separate the different populations of organelles followed by purification techniques (Stasyk and Huber, 2004).

### 4.1.1 Advantages and disadvantages of organelle proteomics

Organelle proteomics is a sensitive and more reliable way of studying proteins than tissue or cellular proteomics, since it allows us to study proteomics of small volume of isolated organelles, which are enriched in high protein content (Au et al., 2007). Organelle proteomics allow for precise protein localisation inside the cell and is linked to organelle function. Thus, organelle proteomics provides a unique opportunity to link proteomics data with subcellular functional unit (i.e. organelle) (Brunet et al., 2003).

On the other hand, contamination of organelle samples is a main problem in organelle proteomics studies (Au et al., 2007).

# OBJECTIVES

Paiement and colleagues (Paiement et al., 1992) found that the microinjection of endoplasmic reticulum (ER) membranes from adult rat liver into blastomeres of Xenopus laevis embryos led to inhibition of cellular division in the blastomeres, while the microinjection of endoplasmic membranes from liver nodules of rats treated with aflatoxin B1 or from livers undergoing regeneration after partial hepatectomy did not. These membrane transplantation studies suggested the presence of cell cycle specific factors in association with ER membranes in adult rat liver, factors which were absent or modified in ER membranes from liver tumors. These microinjections results led to the consideration that the protein composition of ER is different in normal non-dividing cells compared with that of ER from rapidly dividing tumor cells. We have now used mass spectrometry to examine the difference between these two types of ER and to try to explain why ER from rapidly dividing tumor cells are more compatible with cytoplasmic environment of frog blastomeres.

For our study, we used a purified ER from control rat liver and dissected liver tumor nodules. Endoplasmic reticulum membrane fractions enriched in rough and smooth microsomes were isolated by differential centrifugation and sucrose gradient sedimentation from rat liver homogenates (Paiement et al., 2006). First mass spectrometry was used in order to identify the proteome profile of the ER derived from control rat liver and nodular liver, then we used one-dimensional gel electrophoresis in order to separate different proteins followed by immunoblot (using primary and secondary antibodies) to identify and to determine the concentration of ER proteins from control rat liver and from dissected liver tumor nodules. Tables have been generated to compare differentially expressed ER proteins with differentially expressed proteins in nodular liver. Other Table has been generated to identify tyrosine-phosphorylated proteins. Tables were done by using Excel and SPSS software programs, in order to calculate and to identify the overexpressed, underexpressed and equally expressed proteins in tumor ER, means, standard deviation and *P-values* calculations. Graphs have

been generated to calculate the relative concentration of novel proteins, folding proteins, peptide loading complex and proteasome subunits. Densitometry was used to determine the relative concentration of protein bands in control and in tumor ER, those bands that were detected initially by immunoblot.

Our final goal was to study the expression of proteins involved in the process of liver cancer. Consequently, we hope that our work can serve to select the interested proteins for further specific isolated experiments to study in details the related underlying signaling pathways and the corresponding cytosolic and/or nuclear interactions.

The main objectives of this thesis were to perform the following:

- Determine the protein composition of ER fractions from control and tumor liver as well as to determine the identity of the overexpressed and underexpressed proteins in tumor ER. Also, to determine the identity of proteins that did not change in expression in tumor ER relative to control liver ER.

- Confirm by immunoblot and densitometry the relative expression of specific proteins in tumor ER and control liver ER.

- Compare relative concentrations of specific proteins in both ER and cytosolic fractions from dissected liver tumor nodules with similar fractions from control rat liver.

- Use bioinformatics tools to determine the unknown proteins based on protein domain analysis.

# AUTHOR'S CONTRIBUTION

Certain results shown in this thesis had been presented in scientific meetings as follows:

**1 Posters**

- **Roy L., Abdou E., Thibault G., Hamel N., Taheri M., Lanoix J., Kearney R., Paiement J.** Molecular machines of the endoplasmic reticulum cancer. 2nd IRCM Meeting on Systems Biology: Molecular Networks. Montreal, Quebec, Canada. March 11-12, 2008.

- **Abdou E., M Taheri M., Roy L., Thibault G., Servant F., Kearney R., Paiement J.** Carcinome hépatocellulaire: composition protéique du Réticulum endoplasmique. The 24th Annual Scientific Day, Department of Pathology and Cell Biology, University of Montreal, Canada. May 2007.

- **Abdou E., Taheri M., Roy L., Thibault G., Boismenu D., Hayes J., Servant F., Kearney R., Paiement J.** The Proteome of the Endoplasmic Reticulum in Cancer Molecular Biology of the Cell. American Society of Cell Biology. December Supp., 2006.

**2 Article (under preparation)**

- L. Roy, E. Abdou, G. Thibault, N. Hamel, M. Taheri, R. Kaerney and J. Paiement. Quantitative proteomics analysis of the endoplasmic reticulum in hepatocellular carcinoma: An organelle perspective on cancer.

# MATERIALS AND METHODS

## 1 Induction and characterization of rat liver tumors

The animal model was obtained according to procedure previously described by Paiement and colleagues (Paiement et al., 1992) as follows:

### 1.1 Induction of rat liver tumors [1]

- Male Fischer rats (F344, Charles River Canada Inc., St-Constant, Quebec, Canada) weighing 150 ± 10 g were maintained on rat chow (Prolab Agway, Charles River Canada Inc., St-Constant, Quebec, Canada) for 1 week before the start of the experiment. They were kept in a conventionally maintained animal facility (2 animals / cage) under conditions of controlled temperature (22 ± 2°C), humidity (50 ± 10%) and lighting (12-h-light: 12-h-dark daily cycle). Water and food were available ad libitum. Care of the animals conformed to guidelines established by the Canadian Council on Animal Care (Ottawa, Ontario).

- Fifty rats were separated into two groups. Forty rats in group I were feed aflatoxin B1 (AFB1) (Sigma Chemical Co., St. Louis, Missouri, USA). The rats were treated with AFB1 according to Butler and colleagues (Butler et al., 1969), but with the following modifications. Aflatoxin B1 was first dissolved in dimethyl sulfoxide and then provided in a continuous supply in the drinking water at a concentration of 1 μg/ml in darkened bottles. Group II rats were provided with water containing the same amount of dimethyl sulfoxide as provided in group I. Water containing AFB1 or vehicle alone was prepared fresh each week and stored in a cold room (4°C) until needed; it was usually changed 3 times / week, for a total of 55 weeks. This 55-week period represented the total number of days during which rats were treated with AFB1.

## 1.2 Characterization of rat liver tumors [1]

- Rats were starved 48 h before they were killed (Eriksson et al., 1983). At different times after initiation of treatment, livers were recovered and placed immediately in ice-cold sodium immidazole buffer (pH 7.4) containing 0.25 M sucrose. Small fragments of tissue were dissected from the edge of the median lobe and placed in fixative and the remaining mass of liver (≈ 15 g) was used for subcellular fractionation.

- Light and electron microscope studies on the same tissues that were employed to prepare subcellular fractionations. Hence, tissue pieces were fixed by immersion and to maximize fixation efficiency, a fixation and embedding protocol established for use with developing embryos was used (Kalt and Tandler, 1971). Histopathological diagnoses were based upon criteria described by the committee on histologic classification of laboratory animal tumors, Institute of Laboratory Animal Resources (Resources, 1980).

- To determine the extent of cell proliferation occurring after various proliferative stimuli, a single injection of [$^3$H] thymidine (New England Nuclear, Canada; sp.act., 70 Ci/mmol; 1 Ci = 37 GBq) was given intrapertioneally (250 μCi) I h before sacrifice. Pieces of liver (1-3 mm) were obtained and immersed in the fixative of (Kalt and Tandler, 1971). Following dehydration and embedding in paraffin, sections (5 μm thick) were cut serially, put on slides and stained with hematoxylin and eosin. The sections were coated with Kodak NTB2 emuslion and processed for radioautographic analysis (Kopriwa and Leblond, 1962).

- Light microscope radioautography was carried out to determine the [$^3$H] thymidine-labeling index of hepatocytes in tumor-bearing rats. The labeling indices for cells in tumors were compared with those of surrounding liver tissue. Results from 2 different experiments showed higher labeling indices for cells within tumors (4.2- to 10-fold greater [$^3$H] thymidine incorporation). The higher labeling indices found in the nodules indicates the higher rate of cell proliferation within these liver regions.

- Macroscopic analysis of liver from rats treated with AFB1 for up to 45 weeks revealed the presence of multiple white foci (< 5mm). At 51 weeks after initiation of treatment, 2 rats were sacrificed. The liver of 1 rat displayed small foci (< 5mm) and the liver of the other rat revealed the presence of large nodules (> 20 mm). The livers of rats examined 52 weeks or later after initiation of treatment with AFB1 all contained large nodules, several measuring > 20 mm in diameter.

- Light microscopy was carried out on the livers of control and AFB1 treated rats. Control rat liver and dissected liver tumor nodules were fixed in Bouin's Fixative and embedded in paraffin. Ten microns serial sections were cut and stained with Haematoxylin and Eosin. Livers from control rats revealed a normal histology; hepatocytes were mainly arranged in single layers or cords. Livers from rats treated with carcinogen for periods from 21 to 51 weeks contained small foci measuring < 5mm in diameter and had a different histology from that of normal liver. The foci often contained cells with increased amounts of lipid and exhibited different tinctorial properties. The livers of rats examined after treatment with carcinogen for > 51 weeks all contained multiple large nodules measuring > 5mm in diameter. These consisted of cell organizations classified as typical for trabecular hepatocellular carcinoma as well as for glandular hepatocellular carcinoma. Some nodules contained mixtures of these 2 types of tissue organizations and some contained an undifferentiated tissue organization.

In summary, the histological features of the livers of these experimental animals were essentially similar to those described previously for rat liver tumors induced by AFB1 (Butler and Jones, 1978; Jones and Butler, 1978).

## 2 Preparation of subcellular fractions [1]

All subcellular fractions used in the study were derived from adult rat control liver and dissected liver tumor nodules of adult rat AFB1-induced HCC as described before. The macroscopic appearance of a rat liver with associated tumor nodules is shown in Figure 2. The steps were summarized as shown in Figure 3. These fractions include microsomal (smooth and rough) and cytosolic fractions.

Subcellular fractions isolation and characterization was done based on well-established protocols (Paiement et al., 2006). The protocol used can be summarized as follows:

- Briefly, minced rat liver tissue was homogenized. The homogenate was later on submitted to a series of differential centrifugations to allow separation of different cellular components and total microsomal fractions.

- Total microsomes, containing membrane derivatives from different sources (RER and SER), were resuspended in 1.38 M sucrose and placed under 3 steps gradient of 1 M, 0.86 M and 0.25 M sucrose, and centrifuged (using Beckman SW 60 rotor at 300 000g for 1 hour).

- The rough microsomal faction was extracted from the residual pellet below the step gradient. It was then either washed with 4 mM imidazole buffer (pH 7.4) containing 0.25 M sucrose.

- The smooth microsomal fraction, which includes both smooth microsomes and low-density rough micrososmes, was extracted from the upper half of the 1.38 M sucrose step gradient. This fraction was washed once and resuspended in sucrose.

- The cytosolic fractions were obtained from the supernatants remaining after sedimentation of total microsomes at 100 000 g. Hence, the name S100 for the cytosolic fractions used in our study.

- Protein concentrations were determined using the Lowry method (Lowry et al., 1951) with the bovine serum albumin as the standard.

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[1] Line Roy was responsible for treatment of rats with aflatoxin to induce rat liver tumors, was involved in the characterization of the liver tumors and did the subcellular fractionation.

**FIGURE 2: Dissected liver tumor from rat treated with aflatoxin B1.** Photomicrograph show isolated livers after aflatoxin treatment. The asterisk shows a large liver tumor nodule. Such nodules were later on subjected to dissection and extraction of the subcellular fractions used for the Mass spectrometry proteomic analysis and immunoblot experiments.

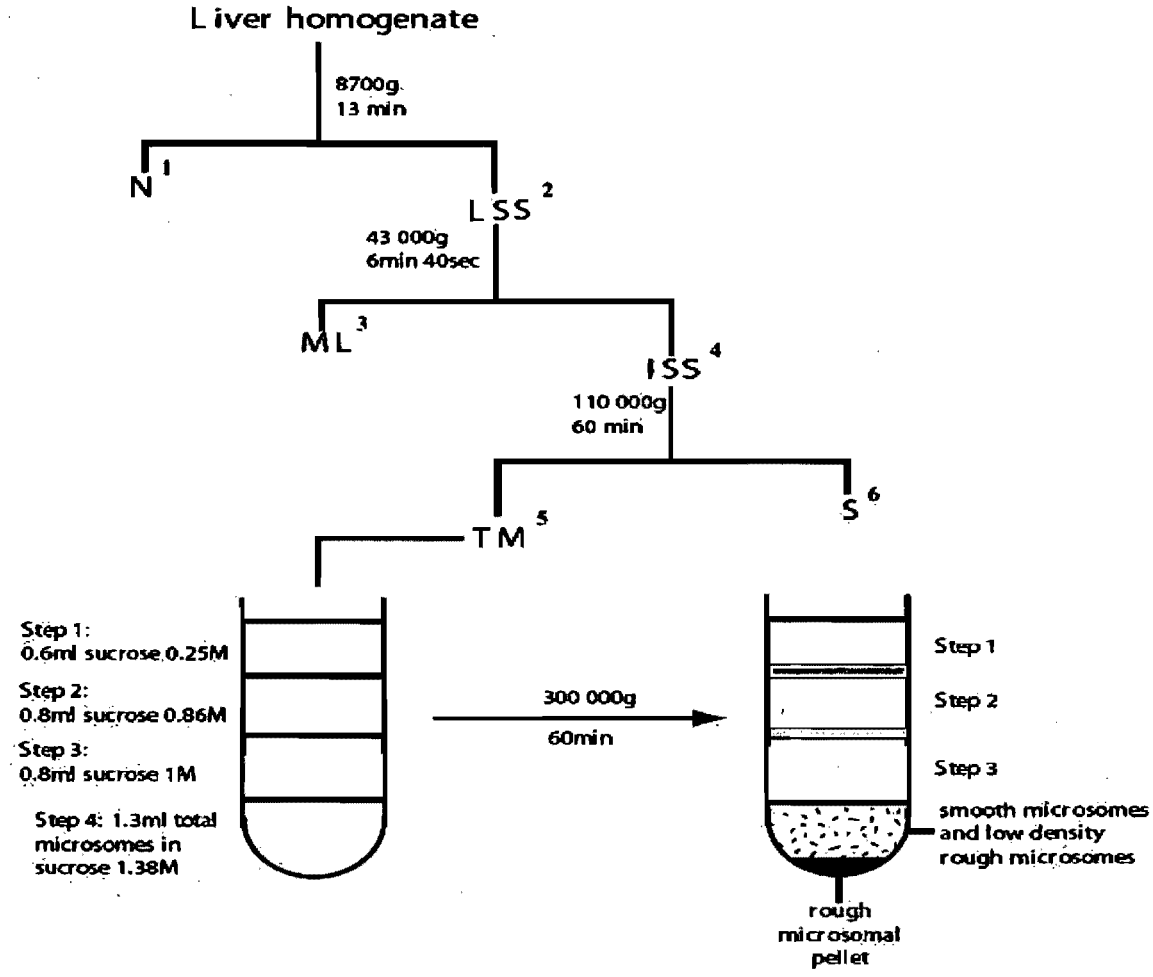


**FIGURE 3: Flow diagram summarizing the fractionation protocol used to prepare ER subfractions from control rat liver and from dissected liver tumor nodules.** Fractionation protocol used to purify ER derivatives is composed of two main steps where the liver homogenate was subjected to differential centrifugation followed by gradient centrifugation using a sucrose step gradient (Paiement et al., 2006).

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[1] N: Nucleus

[2] LSS: Low-speed supernatant.

[3] ML: ML fraction, containing lysosomes and mitochondria.

[4] ISS: Intermediate-speed supernatant

[5] TM: Total microsomes.

[6] S: supernatant

# 3 Mass spectrometry

The identification of proteins based on mass spectrometry analysis has opened new opportunities for the study of proteins of organelles like ER (Yates et al., 2005). Mass spectrometry can show the sequence of a peptide; this process passes by several steps starting with organelle sampling, purification analysis, gel separation and digestion gel cutting. This is followed by accumulation of mass spectrometry, peptide identification (by using Mascot software program that does the peptide and protein identification by comparing the mass spectrometry raw file results to all theoretical peptide spectra calculated to obtain a peptide sequence), scoring and clustering (protein list) and finally annotation and interpretation (proteome). Figure 4 briefly summarizes the steps involved.

## 3.1 Protein separation, mass spectrometry and data analysis

Samples (from 4 different subcellular fractionation experiments) were solubilized in Laemmli buffer and 75 µg proteins of each sample were loaded on 5.2 cm 1D SDS [1] - PAGE with 7-15% gradient acrylamide. The gel was stained with Coomassie Brilliant blue G (Sigma, Oakville, Ontario, Canada). The full lane has then been subjected to automated band excision, to generate 28 bands per lane. Following transfer to a 96-well tray, proteins from gel bands were subjected to reduction, alkylation and in-gel tryptic digestion by automation in a MassPrep Workstation (Micromass, Manchester, UK) as previously described (Wasiak et al., 2002). Briefly, gel bands were cut into 1 $mm^3$ gel pieces and put in separate wells of a 96-well sample tray. Gel pieces were then prewashed by two 10 minute incubations in 100 µl HPLC [2] grade water. All steps up to trypsin addition were done at 37°C. After removal of water, gel pieces were destained by incubating 10 minutes in 50 µl of 100 mM ammonium bicarbonate followed by addition of 50 µl of 100% acetonitrile and incubating an additional 10 minutes. Pieces were then incubated for an additional 5 minute in 100% acetonitrile and liquid removed. Destained gel pieces were then sequentially reduced and alkylated by incubating 30 minutes in 50 µl of 10 mM dithiothreitol, adding 50 µl of 55 mM iodoacetamide, incubating 20 minutes, adding 100 µl of 100% acetonitrile and incubating 5 minutes. Liquid was

removed and gel pieces were washed by incubating 10 minutes in 50 µl of 100 mM ammonium bicarbonate, adding 50 µl of 100% acetonitrile, incubating 5 minutes and removing liquid. After dehydration, gel pieces were dried 30 minutes at 37°C. Proteins were in-gel digested by adding 25 µl of trypsin (6 ng/µl in 50 mM ammonium bicarbonate, Promega sequencing grade modified trypsin, cat number V511A) and incubating 30 minutes at room temperature followed by 4 hours 30 minutes at 37°C. To extract peptides, 30 µl of a mix containing 1% formic acid and 2% acetonitrile was added, incubated 30 minutes at room temperature, and 30 µl of the liquid was transferred into a new cooled (10°C) tray. Two additional extractions are performed. 12 µl of a mix of 1% formic acid and 2% acetonitrile and 12 µl of 100% acetonitrile were added, incubation continued 30 min and 15 µl removed and pooled with the first extraction. This step was repeated with the transfer of an additional 15 µl of extract yielding a final volume of peptide extract of 60 µl at 0.54% formic acid and 15.9% acetonitrile.

Extracted peptides were then subjected to mass spectrometry. The 96-well sample tray was kept in a Micro Well-plate Sampler (Agilent 1100 Series). Prior to injection, the precolumn (Zorbax 300SB-C18, 5 mm X 0.3, 5 µm) installed on the 6 port Rheodyne valve of the Column Compartment Module, was conditioned with water containing acetonitrile (5%) and formic acid (0.1%) supplied by a Isocratic pump (Agilent 1100 series) set at a flowrate of 15 µL/min. A volume of 20 µL of the tryptic digest solution was injected on the precolumn at 15 µL/min and the sample was washed for 5 min while the flow through was evacuated to waste. The valve was actuated and the pre-column was back-flushed to the 75 mm i.d PicoFrit column (New Objective, Woburn, MA) (filled with 10 cm of BioBasic C18 packing, 5 mm, 300 Å) by the acetonitrile gradient (5–70% acetonitrile/0.1% formic acid) supplied by the Agilent series 1100 Nanopump at 200 nL/min. Solvent A was water (formic acid 0.1%) and solvent B was acetonitrile:water:formic acid (95:5:0.1). The linear gradient was started after the washing step. The mass spectrometer was a QTOF $^{3}$ Micro from Waters Micromass equipped with a Nanosource modified with a nanospray adapter (New Objective, Woburn, MA) to hold the PicoFrit column tip near the sampling cone. The capillary voltage was adjusted to get the best spraying plume at 35% B. MS survey scan was set to

1 s (0.1 s interscan) and recorded from 350 to 1600 m/z. In a given MS Survey scan, all doubly and triply charged ions with intensity higher that 25 counts were considered candidates to undergo MS/MS fragmentation. From these, the strongest one was selected. MS/MS acquisition would stop as soon as the total ion current would reach 2800 counts/second or after a maximum time of 4 s. MS/MS scan was acquired from 50 to 1990 m/z, scan time was 1.35 s and interscan was 0.15 s. A second precursor ion would be selected from the following MS Survey scan. The doubly and triply charged selected ions were fragmented with the following preprogrammed collision energies: (i) For doubly charged ions, the collision energies are 25 eV for the range 400 to 653 m/z, 26 eV for the range 653 to 740 m/z, 28 eV for the range 740 to 820 m/z, 32 eV for the range 820 to 1,200 m/z, and 55 eV for the range 1,200 to 1,600 m/z. (ii) For triply charged ions, the collision energies are 14 eV for the range 435 to 547 m/z, 19 eV for the range 547 to 605 m/z, 24 eV for the range 605 to 950 m/z, and 35 eV for m/z higher than 950 m/z.

Mass spectrometric data were acquired by employing the Data Directed Analysis feature available on MassLynx (Micromass) operating software with a 1, 1, 4 duty cycle (1 second in MS mode 1 peptide selected for fragmentation, maximum of 4 seconds in MS/MS acquisition mode). MS/MS raw data were transferred from the QTOF Micro computer to a 50 terabytes server and automatically manipulated for generation of peaklists by employing Distiller version 1.1 (http://www.matrixscience.com/distiller.htmls) software with peak picking parameters set at 20 as for Signal Noise Ration (SNR) and at 0.7 for Correlation Threshold (CT). The peaklisted data was then searched against a copy of the National Center for Biotechnology Information (NCBI) non redundant (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) data base (NCBI nrdb January 05, 2007) by employing Mascot (http://www.matrixscience.com) version 2.1.04, and restricting the search to up to 1 missed (trypsin) cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, 0.5 mass unit tolerance on parent and fragment ions, and monoisotopic. The resulting list of peptide identifications was then processed to eliminate those likely to have arised by chance. Thus, only peptide identifications with a probability of occurring by chance of $< 0.05$ were retained (i.e. only

those peptides for which the Mascot Peptide Score was > the Id Score were retained). Validation studies in which spectra were searched against a random data base, showed that a Mascot score at the 5% confidence level was quite conservative; the actual false positive rate was estimated to ~ 1.5% estimated by searching a randomized copy of the database. The search was limited to the Rattus taxonomy.

Mascot results from bands 1 to 28 (based on spectra assigned to tryptic peptide sequences at the 95% confidence level) generated peptide identifications were then linked to the proteins and sorted by protein to produce an initial list of protein identifications. However, this list was quite redundant since about 5% of the spectra match more than one peptide and 40% of the peptides identified occur in more than one protein. Consequently, the protein lists were processed by a grouping algorithm (Kearney et al., 2005) to generate a list of proteins defined by distinct sets of proteins i.e. the minimum number of protein sequences needed to explain the peptides observed.

Unique peptides were peptides that contained at least one or more amino acids that pointed to a single protein. Some proteins in protein families like the cytochrome p450 family of proteins yielded peptides that were identical in amino acid sequence; these peptides were considered as shared peptides. The growing algorithm of Kearney and colleagues (Kearney et al., 2005) attributed shared peptides to specific proteins based on the proportion of unique peptides attributed to the proteins within a protein family that shared the same peptide, for example if protein A of a specific family had 2 unique peptides and protein B of this same family had 4 unique peptides and these proteins shared 6 peptides protein A would be attributed 2 of the shared peptides for a total of 4 peptides for protein A. Four of the shared peptides were attributed to protein B for a total of 8 peptides for protein B. If there are no unique peptides for protein A or protein B but only "shared peptides", they are identified by family name.

The Oncomine (Rhodes et al., 2007) Database was used to compare our rat ER protein expression data with protein expression data from human cancer tissues and normal controls.

[1] SDS: Sodium dodecyl sulfate.

[2] HPLC: High-performance liquid chromatography.

[3] QTOF: Quadrupole–time-of-flight.

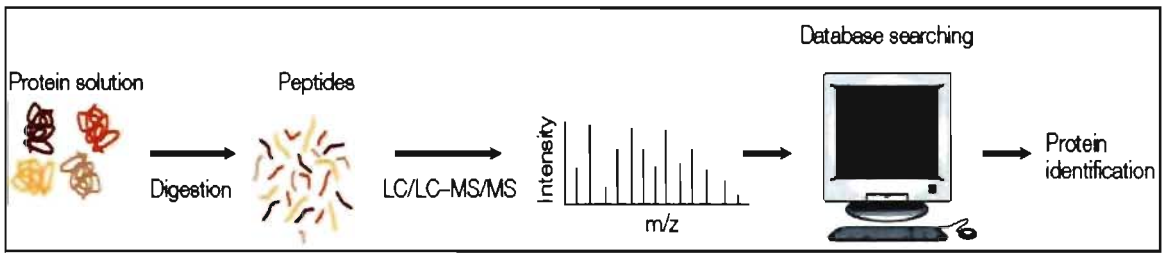


**FIGURE 4: Diagram for simplified process of proteomics.** The tissue is first subjected to fractionation to obtain high purity subcellular fractions. This is followed by enzymatic digestion of the proteins into smaller units (peptides). The total amount of peptides is processed by the mass spectrometer and sorted according to different factors including the mass of the peptide and its charge. The sequence of the peptide is determined by fragmentation. The MS/MS step involves first determination of the mass of the peptide. The dominant ions in the MS spectrum are selected for the transmission through a first analyser, then fragmented in a collision cell and their fragments analysed in a second analyser to produce a MS/MS spectrum. The sequence (amino acid backbone) is determined and the data obtained are fed to a special computer software (Mascot) to obtain a distinct protein identity (From Yates et al., 2005).

## 4 Gel electrophoresis experiments

One-dimensional gel electrophoresis, mini-gel was run in a Bio-Rad Mini-Protean electrophoresis cell (Bio-Rad Laboratories Inc., Hercules, California, USA). This mini-gel was a gradient one composed of resolving gel (RG/ 7-15%) at the bottom and superior/stacking gel (SG/5%). Both types of gels are composed of 6 reagents with different amounts as follows:

- Bidistilled water (5.35 ml (7%), 1.2 ml (15%) SG and 5.55 ml RG.

- Acrylamide mixture (30%) (2.3 (7%) ml, 5 ml (15%) SG and 1.7 ml RG).

- TRIS [1] – HCl [2] (pH (8.8 SG, 6.8 RG) (2.25 (7%, 15 %), ml SG, 2.5 ml RG).

- SDS [3] (10%) (100 µl SG, RG), TEMED [4] (10 µl SG, RG).

- Finally, a freshly prepared APS [5] (10%) (10 µl (7 %, 15 %) SG and 100 µl RG).

The latter 2 reagents are catalysts of gel formation and so are added just before pouring in the gel.

Proteins samples were loaded to a constant quantity of 40 µl/well, Laemmli sample buffer was added to each protein sample except the protein sample standards (since the Laemmli is already included by the manufacturer). Then the prepared samples were boiled for about 5 min to break the disulphide bonds by the reducing agent (ß-mercaptoethanol) found in Laemmli sample buffer and to enhance the protein samples to be charged negatively upon being in contact with SDS during electrophoresis. Each protein sample was loaded in its appropriate gel well. The Bio Rad protein standards used had a range from 250 to 10 kD. To separate the loaded protein samples, electrophoresis (at a constant 200 Volts) was started until migration of proteins in their correspondent wells was completed. This was assured by observing the bromophenol front blue dye (a

component of Laemmli) to be approaching the lower end of glass plates. The bromophenol molecule is a small molecule that migrates rapidly towards the bottom of the gel.

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[1] TRIS: Tishydroxymethylaminomethane

[2] HCl: Hydrochloric acid.

[3] SDS: Sodium dodecyl sulfate.

[4] TEMED: Tetramethylethylenediamine

[5] APS: Ammonium persulfate.

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## 5 Immunoblot (Western blot) experiments

At the end of the gel electrophoresis, the separation of the proteins was achieved. The transfer of the gel proteins to nitrocellulose membrane was achieved by using a Bio-Rad Mini-Transblot cell (Bio-Rad Laboratories Inc., Hercules, California, USA). Blotting on the nitrocellulose membrane, 0.2 µm (Bio-Rad Laboratories Inc., Hercules, California, USA) was successful when we could observe the complete transfer of the Precision Plus Protein Standards, Dual Color (Bio-Rad Laboratories Inc., Hercules, California, USA) on the membrane. Hence, the membrane was placed in a blocking solution (containing 5% milk and 0.2% Tween-20 in PBS [1] 1X pH 7.4) at room temperature for 1 hour. The milk proteins would saturate the free non-specific binding sites on our studied proteins; this could prevent the non-specific antibody reaction and consequently the occurrence of background effect latter on. After this step of non-specific saturation of protein binding sites, the membrane was incubated with the primary antibody (previously diluted in the blocking solution) at 4°C over night. A variety of primary antibodies were used in our studies, these are summarized in Table 1. After 3 times of washings (each for 10 minutes) with the blocking solution, the membrane was incubated with the horseradish peroxidase-

conjugated secondary antibody at room T°C for 1 hour. After another set of 3 washings (each for 10 minutes) with the blocking solution then final wash with bidistilled water, the protein was revealed using a freshly prepared Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer Life Sciences Inc., Boston, Massachusetts, USA). Localisation of the secondary antibodies on the blots was done by using Kodak Biomax ML scientific imaging film (Scientific Imaging Systems Eastman Kodak Company, New Haven, Connecticut, USA).

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[1] PBS: Phosphate buffered saline.

**TABLE 1: List of the primary antibodies used in the immunoblot experiments.** Eighteen antibodies [1] were used. All of them are polyclonal except for the anti-CYP p450 2C11 and the anti- phosphotyrosine which are monoclonal. They have been used to probe microsomal and/or cytosolic fractions derived from control rat liver and dissected liver tumor nodules.

| Antibody | Poly/Mono clonal | Animal | Company |
|---|---|---|---|
| Anti-rAFAR 1 | Polyclonal | Rabbit | kindly provided by John Hayes, University of Dundee, Dundee, Scotland |
| Anti-Bip/GRP 78 | Polyclonal | Rabbit | ABCAM Inc., Cambridge, MA, USA |
| Anti-ATP citrate lyase | Polyclonal | Rabbit | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-CYP 450 2C11 | Monoclonal | Mouse | Tekniscience Inc., Terrebonne, Quebec, Canada |
| Anti-CYP 450 4A [2] | Polyclonal | Rabbit | Tekniscience Inc., Terrebonne, Quebec, Canada |
| Anti-NADPH-CYP p450 reductase | Polyclonal | Sheep | Chemicon International, Inc., Temecula California, U.S.A |
| Anti-eEF 2 | Polyclonal | Rabbit | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-eIF 2α | Polyclonal | Rabbit | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-GAS 2 | Polyclonal | Rabbit | kindly provided by Claudio Schneider Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, Trieste, Italy |
| Anti-LRP/MVP | Polyclonal | Goat | Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA |
| Anti-PA2G4/EBP 1 | Polyclonal | Chicken | Tekniscience Inc., Terrebonne, Quebec, Canada |
| Anti-PABP 1 | Polyclonal | Rabbit | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-phosphotyrosine | Monoclonal | Mouse | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-RER | Polyclonal | Rabbit | (Paiement and Roy, 1988) |
| Anti-phospho-S6 ribosomal protein [3] | Polyclonal | Rabbit | New England Biolabs Ltd. Pickering, Ontario, Canada |
| Anti-hnRNP K | Polyclonal | Rabbit | Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA |
| Anti-VCP/P97 | Polyclonal | Rabbit | Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA |
| Anti-YB 1 | Polyclonal | Goat | Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA |

[1] Anti-rAFAR 1 (anti-rat aflatoxin B1 aldehyde reductase member 1), anti-Bip/GRP 78 (anti-immunoglobulin heavy chain-binding protein/ anti-78 kDa glucose-regulated protein precursor), anti-ATP citrate lyase (anti-denosine triphosphate), anti-CYP 450 2C11 (anti-cytochrome p450 family protein), anti-CYP 450 4A (anti-cytochrome p450 family protein), anti-NADPH-CYP p450 reductase (anti-nicotinamide adenine dinucleotide phosphate-cytochrome p450 reductase), anti-eEF 2 (anti-eukaryotic translation elongation factor 2), anti-eIF 2α (anti-eukaryotic translation initiation factor 2, subunit 1 alpha), anti-GAS 2 (anti-growth arrest-specific protein 2), anti-LRP/MVP (anti-lung resistance related protein/anti-major vault protein), anti-PA2G4/EBP 1 (anti-proliferation-associated protein 2G4/anti-ErbB3 binding protein 1), anti-PABP 1 (anti-polyadenylate-binding protein 1/anti-poly(A)-binding protein 1), anti-phosphotyrosine, anti-RER (anti-rough endoplasmic reticulum), anti-phospho-S6 ribosomal protein, anti-hnRNP K (anti-heterogeneous nuclear ribonucleoprotein complex K), anti-VCP/P97 (anti-valosine-containing protein/anti-p97) and anti-YB 1 (anti-Y box protein 1).

[2] The primary antibody anti-CYP 450 4A recognises CYP 450 4A1, CYP 450 4A2 and CYP 450 4A3.

[3] The primary antibody anti-phospho-S6 ribosomal protein detects ribosomal S6 protein when it is only phosphorylated.

## 6 Densitometry calculations

Densitometry was carried out to determine the relative concentration of proteins in control rat liver and dissected liver tumor nodules. Kodak films from immunblots were scanned and analyzed using densitometric computer software namely Image J and Scion Image. The basic steps used are summarized as follows:

### 6.1 ImageJ (National Institutes of Health, USA) [1]

- Immunoblot experiments were done on subcellular fractions as described before.

- The immunoblots obtained for different fractions were filmed using Kodak films then scanned.

- NIH ImageJ version 1.38x densitometry software program was used to obtain the density of the scanned immunolabeled bands. The software created tracings with peaks that has height and area information, which corresponded with relative stain intensity. Image J would calculate the amount for each tracing. This data was then transferred to Microsoft Office Excel for comparison in order to obtain at the end the relative quantity of each of these fractions.

- Finally, the curves and the measurements obtained were saved for our own records on the computer.

- Statistics was used to confirm different amounts of proteins determined by densitometry.

**6.2 Scion Image** (Scion Corporation, Maryland, USA) [2]

Scion Image software program was also used. It is based on the same concept as ImageJ but the images must be initially in a «TIF» format, to work with this program. In addition, the background for the ECL signal was calculated for each lane containing the selected band (s) and subtracted from each selected immunolabeled band in this lane in order to get the real densitometric value for the studied band.

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[1] http://rsbweb.nih.gov/ij/docs/pdfs/ImageJ.pdf.

[2] http://www.scioncorp.com

# 7 Statistical data analysis: Ratio and *P-value* calculations

We carried out statistical analysis to compare the number of peptides found in ER fractions from control rat liver with fractions from dissected liver tumor nodules. Ratios of peptide counts were done as described below.

## 7.1 Calculation of ratios of total peptides

The equation summarizing the calculation process of ratio of total peptides for a selected protein:

$$R\ \frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$$

R= Ratio of the sum of peptides of a selected protein in SMNod divided by the sum of peptides of the same protein in SMCtl.

SMNod= Smooth microsomes from control rat liver.

SMCtl= Smooth microsomes from dissected liver tumor nodules.

N1= Number of total peptides in MS experiment number 1.

N2= Number of total peptides in MS experiment number 2.

N3= Number of total peptides in MS experiment number 3.

N4= Number of total peptides in MS experiment number 4.

4N= Number of total peptides in total of 4 MS experiments.

## 7.2 Calculation of *P-value* (test of significance for differentially expressed proteins)

*P-value* refers to difference between the means of the peptides between tumor and control ER fractions. Data sets obtained via Excel Microsoft office program were analyzed using SPSS (version 16.0, SPSS Science) [1]. Paired-samples T-Test was used to determine *P-value*. The means of the total peptides for a protein were considered

significantly different between control and treated when they gave a *P-value* equal to or less than 0.05.

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[1] http://www.spss.com/scienceproducts.htm

# RESULTS

Most of the comparison of proteins was done using smooth microsomes. However, in certain cases data obtained using rough microsomes from dissected liver tumor nodules are also included for comparison with results obtained using smooth microsomes of the same origin.

## PART A: Mass spectrometry (MS) analysis

### A.1 Known proteins

#### A.1.1 Comparison of our results with the results reported in the cancer literature

We compared differentially expressed proteins detected in our study with differentially expressed proteins that had been described in cancer literature. Differentially expressed proteins may be either proteins overexpressed in tumor ER or proteins underexpressed in tumor ER. We have compared our mass spectrometry results with recent human liver cancer literature. The cancer literature has identified a number of proteins as being overexpressed or underexpressed in tumor tissues. We have detected the same proteins to be overexpressed or underexpressed in ER fractions from rat liver tumor nodules. Our results are comparable to those reported in the cancer literature based on the quantitative definition for differential expression. In our study, we indicate which proteins are differentially expressed and point out which of these have already been reported as being differentially expressed in the human cancer literature. The exact amounts of overexpression or underexpression cannot be compared because the quantitative procedures used by the cancer literature were considerably different from ours. The cancer literature used different techniques; like two-dimensional gel electrophoresis and mass spectrometry (Blanc et al., 2005; Takashima et al., 2006; Yokoyama et al., 2004), proteomics and serologic analysis of recombinant cDNA

expression libraries (Takashima et al., 2006). In addition, our data reflects a comparison of organelle proteomics versus cell and tissues proteomics as well as a comparison of proteomics versus DNA microarray (Simpson and Dorow, 2001). We first looked at overexpressed then underexpressed proteins. Key point to mention is that all literature studied cells and tissues, while our study was done at the level of an organelle, the ER. Overexpressed and underxpressed data are shown in Table forms (Tables 2-A and 2-B respectively). We also described the main function of each protein shown in each Table.

### A.1.1.1 Overexpressed proteins reported in cancer literature

Overexpressed proteins can be defined in two ways. First, as regard our study, they are defined, as the proteins with total number of peptides in the tumor ER were more than the total number of peptides in control ER fractions. However in the literature used as reference in this Table the overexpressed proteins were defined for example by using two-dimensional gel electrophoresis maps, the ratio of spot intensities of HCC and non-tumor tissues was used, a positive ratio indicated overexpression in the tumor (Blanc et al., 2005; Yokoyama et al., 2004). However, different study methods were used as follows: in one study, immunoblot was used to identify immunoreactive spots with stronger staining intensity in tumoral tissues than in corresponding normal liver tissues (Takashima et al., 2006) and in a second study reports of human proteomic analyses of tumor tissues and non-tumor tissues sampled from HCC were collected, in order to classify the proteins into different functional categories (Kuramitsu and Nakamura, 2006).

Our results were compared with four different studies on human HCC. Table 2-A shows the results of our comparison.

**TABLE 2-A: Overexpressed proteins in ER of HCC: Comparison with other studies on HCC.** The overexpression (ratio, R) [1] of the total peptides of specific proteins of smooth microsomes (SM) from dissected liver tumor nodules (Nod) as regard from control rat liver (Ctl) [SMNod/SMCtl] from 4 repeated mass spectrometry experiments (4N) were calculated.

| Protein | Function [2] | Total peptides SMNod/SMCtl | Overexpression SMNod/SMCtl | Reference (s) |
|---|---|---|---|---|
| Glutamate-cysteine ligase | Metabolism | 9.5/0 | - | (Blanc et al., 2005) |
| Vimentin | Cytoskeleton | 1.5/0 | - | (Kuramitsu and Nakamura, 2006) |
| Nucleophosmin | Protein synthesis | 1.3/0 | - | (Blanc et al., 2005; Kuramitsu and Nakamura, 2006) |
| Transaldolase | Metabolism | 10.3/1.3 | 8.2 | (Blanc et al., 2005) |
| Vesicle amine transport protein 1 | Traffic | 2/0.25 | 8 | (Blanc et al., 2005) |
| Annexin II | Lipid metabolism | 7.5/1 | 7.5 | (Kuramitsu and Nakamura, 2006) |
| Apolipoprotein A-I | Secretory protein | 5.5/1 | 5.5 | (Blanc et al., 2005) |
| Annexin V | Lipid metabolism | 7/2.3 | 3.1 | (Kuramitsu and Nakamura, 2006) |
| hnRNP K | mRNA metabolism | 14.5/6.3 | 2.3 | (Blanc et al., 2005; Kuramitsu and Nakamura, 2006) |
| Eukaryotic translation initiation factor 5A-1 (eIF 5A) | Protein synthesis | 4.5/2.5 | 1.8 | (Kuramitsu and Nakamura, 2006) |
| Elongation factor 2 | Protein synthesis | 34.3/19 | 1.8 | (Kuramitsu and Nakamura, 2006) |
| rAFAR 2 | Detoxification | 2.5/1.5 | 1.7 | (Kuramitsu and Nakamura, 2006) |
| HSP 60 | Folding protein | 5/3.3 | 1.5 | (Blanc et al., 2005; Kuramitsu and Nakamura, 2006) |
| Glyceraldehyde-3-phosphate dehydrogenase | Metabolism | 25.5/19.5 | 1.3 | (Takashima et al., 2006) |
| Heat shock protein 8 (HSP 70) | Folding protein | 28.2/23.6 | 1.2 | (Takashima et al., 2006; Yokoyama et al., 2004) |

[1] R $\frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$

[2] Functional annotations were obtained primarily from Gilchrist and colleagues (2006).

Table 2-A, shows proteins that we observed to be overexpressed in tumor ER and which were also reported to be overexpressed in the cancer literature. Many of these proteins are cytosolic proteins (Swiss-prot database, http://ca.expasy.org/sprot/). These results suggest that many studies reported in the literature detect very few ER proteins in tumor cells and tissues. We have chosen to discuss details of the following proteins for two reasons. One they are highly overexpressed in our study and two they have been reported by others either to play an important role in ER fraction or to be differentially expressed in cancer. Proteins are statistically significant when *P-value* is $\leq 0.05$ (this means that the total number of peptides for a given protein was determined to be statistically different when compared between tumor and control membranes. A statistical difference was considered significant when the comparison of the means of the total peptides gave a *P-value* is $\leq 0.05$ by paired-samples T tests). Overexpressed proteins of note in cancer literature are as follows:

- Transaldolase plays a role in metabolism. It derives this from being a key enzyme in the pentose phosphate pathway (non-oxidative branch). This metabolic pathway enzyme plays a role in glucose metabolism at the level of the ER (Boren et al., 2006; Bublitz and Steavenson, 1988; Heinrich et al., 1976). In Table 2-A, it is the most overexpressed protein in tumor ER (8-fold increase).

- Annexin II is an immunohistochemical tumor marker studied in cancer, examples include high-grade prostate cancer (Banerjee et al., 2003) and renal cell carcinoma (Zimmermann et al., 2004). It is statistically significant ($P = 0.02$).

- Heterogeneous nuclear ribonucleoprotein K (hnRNP K) belongs to the hnRNP family (Habelhah et al., 2001). The hnRNPs are mRNA-binding proteins and are involved in mRNA metabolism in the cytoplasm and nucleus (Lavoie and Paiement, 2008). Hence, this family plays a role in many processes that compose gene expression (Palaniswamy et al., 2006). In our data, hnRNP K is found in ER. In colorectal adenocarcinoma hnRNP K had been studied as a potential new marker in cancer (Klimek-Tomczak et al., 2006). It is statistically significant ($P = 0.04$).

- Rat aflatoxin B1 aldehyde reductase member 2 (rAFAR 2) is a well-known cytosolic protein. It belongs to the aldo-keto reductase superfamily and is involved in aflatoxin detoxification (Knight et al., 1999; Praml et al., 2003; Zeindl-Eberhart et al., 2001). It is normal for us to observe overexpression of rAFAR 2 in our study because rat was treated with aflatoxin B1. Why this protein was reported overexpressed in human HCC (Kuramitsu and Nakamura, 2006) is not known.

- Vimentin is an intermediate filament cytoskeleton protein found in the cytoplasm (Lazarides, 1982). It is considered an immunohistochemical marker for HCC metastases (Hu et al., 2004).

- Nucleophosmin is involved in mRNA metabolism (Palaniswamy et al., 2006). It acts as an inhibitor of apoptosis since it naturally inactivates p53 suppressor tumor protein leading to tumor progression (Mai et al., 2006; Maiguel et al., 2004). It is proposed as a new potential immunohistochemical marker for human HCC (Yun et al., 2007).

- Elongation factor 2 (EF 2) is involved in protein synthesis (Riis, 1996) and RNA metabolism (Kaneda et al., 1984). Phosphorylation of EF 2 plays a role in the proliferation of both normal and malignant glia cells in rat (Bagaglio and Hait, 1994).

In summary, we detected many overexpressed proteins. Some of these proteins are expected like proteins that are involved in protein synthesis, but some of these are

unexpected like annexin and transaldolase. The unexpected proteins could be either contamination of ER fractions or they could be associated with ER for undefined reason.

### A.1.1.2 Underexpressed proteins reported in cancer literature

Underexpressed proteins can be defined in different ways. In our study they are defined as the proteins showing fewer peptides in the tumor ER compared with control ER. Other studies have quantified proteins using two-dimensional PAGE. In such studies protein spot intensities were quantified and underexpressed proteins defined as those proteins in tumor samples that had lower intensities compared with those from control samples (Blanc et al., 2005; Chignard et al., 2006; Takashima et al., 2006; Yokoyama et al., 2004).

Our results were compared with four different studies on human HCC. Table 2-B shows the results of our comparison.

**TABLE 2-B: Underexpressed proteins in ER of HCC: Comparison with other studies on HCC.** This Table is based on the same idea as (Table 2-A). The underexpression (ratio, R) [1] of the total peptides of smooth microsomes (SM) from control rat liver (Ctl) as regard to those from dissected liver tumor nodules (Nod) [SMNod/SMCtl] from 4 repeated mass spectrometry experiments (4N) were calculated.

| Protein | Function [2] | Total peptides SMNod/ SMCtl | Underexpression SMNod/ SMCtl | Reference (s) |
|---|---|---|---|---|
| Phosphoenol Pyruvate carboxykinase | Carbohydrate metabolism | 0/2.8 | 0 | (Blanc et al., 2005) |
| Glutathione peroxidase | Detoxification | 0.5/4.5 | 0.1 | (Kuramitsu and Nakamura, 2006) |
| Phenylalanine hydroxylase | Metabolism | 0.3/2.3 | 0.1 | (Blanc et al., 2005) |
| Ketohexokinase | Carbohydrate metabolism | 0.8/3.8 | 0.2 | (Blanc et al., 2005; Kuramitsu and Nakamura, 2006; Yokoyama et al., 2004) |
| 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 | Lipid metabolism | 0.5/2.3 | 0.2 | (Blanc et al., 2005) |
| Betaine homocysteine methyltransfearse | Metabolism | 8.5/46.9 | 0.2 | (Blanc et al., 2005) |
| Argininosuccinate synthase | Metabolism | 5/31 | 0.2 | (Kuramitsu and Nakamura, 2006) |
| 3-hydroxyanthranilate 3,4-dioxygenase | Metabolism | 1/3.3 | 0.3 | (Blanc et al., 2005) |
| Fructose-1,6-biphosphatase | Carbohydrate metabolism | 2.8/10 | 0.3 | (Blanc et al., 2005) |
| Fatty acid binding protein | Lipid metabolism | 6/18.5 | 0.3 | (Blanc et al., 2005) |
| Fumarylacetoacetase | Metabolism | 6/13.8 | 0.4 | (Kuramitsu and Nakamura, 2006) |
| Arginase I | Metabolism | 16.3/40.3 | 0.4 | (Chignard et al., 2006; Kuramitsu and Nakamura, 2006; Yokoyama et al., 2004) |
| Regucalcin | Signalisation | 6/11.5 | 0.5 | (Blanc et al., 2005) |
| Formiminotransferase cyclodesaminase | Metabolism | 8/14.8 | 0.5 | (Blanc et al., 2005) |
| Annexin VI | Lipid metabolism | 6.3/12.8 | 0.5 | (Blanc et al., 2005) |
| Aldehyde dehydrogenase 9 | Metabolism | 0.5/1 | 0.5 | (Blanc et al., 2005) |
| Adenosyl homocysteinase | Metabolism | 3/5.5 | 0.5 | (Blanc et al., 2005; Kuramitsu and Nakamura, 2006) |
| Aldolase B | Carbohydrate metabolism | 12.3/20.8 | 0.6 | (Kuramitsu and Nakamura, 2006) |
| Superoxide dismutase | Metabolism | 3.8/5.8 | 0.7 | (Chignard et al., 2006) |
| Triosephosphate isomerase | Carbohydrate metabolism | 5.8/8.3 | 0.7 | (Kuramitsu and Nakamura, 2006) |
| Protein disulfide isomerase | Metabolism of disulfide bonds | 8.8/11.8 | 0.7 | (Kuramitsu and Nakamura, 2006) |

[1] $R = \frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$

[2] Functional annotations were obtained primarily from Gilchrist and colleagues (2006).

Table 2-B, shows proteins we observed to be underexpressed in tumor ER and which were also reported to be underexpressed in the cancer literature. Many of these proteins are cytosolic proteins (Swiss-prot database, http://ca.expasy.org/sprot/). These results suggest that many studies reported in the literature detect very few ER proteins in tumor cells and tissues. We have chosen to discuss details of the following proteins for two reasons. One they are highly underexpressed in our study and two they have been reported by others either to play an important role in ER fraction or to be differentially expressed in cancer. Proteins are statistically significant when *P-value* is $\leq 0.05$ (this means that the total number of peptides for a given protein was determined to be statistically different when compared between tumor and control membranes. A statistical difference was considered significant when the comparison of the means of the total peptides gave a *P-value* is $\leq 0.05$ by paired-samples T tests). Underexpressed proteins of note in cancer literature are as follows:

- Ketohexokinase (fructokinase) is involved in carbohydrate metabolism. In the liver, it catalyses the phosphorylation of fructose to fructose-1-phosphate, the latter will follow the glycolytic pathway (Hwa et al., 2006). In our data, it is detected as an underexpressed protein, this is also the case in a previous study for renal tumor in human (Hwa et al., 2006). It is statistically significant ($P = 0.02$).

- Fructose-1,6-biphosphatase is involved in carbohydrate metabolism. It is a key regulatory enzyme in gluconeogenesis. It catalyses the hydrolysis of fructose-1,6-bisphosphate into fructose-6-phosphate and inorganic phosphate (Ke et al., 1989; Loffler et al., 2001). In our data, it is detected as an underexpressed protein; this is in consistent

with a previous study for HCC in human (Taketa et al., 1988). It is statistically significant ($P = 0.003$).

- Fatty acid binding protein is involved in lipid metabolism (Glatz et al., 2002; Storch and Thumser, 2000). It is a cytosolic protein (Mansbach and Dowell, 2000) which is important for intracellular binding and transport of fatty acids (Storch and Thumser, 2000) from apical membrane of enterocyte to ER (Black, 2007). It has been proposed as an immunohistochemical marker for human HCC (Suzuki et al., 1990). It is statistically significant ($P = 0.02$).

- Regucalcin is a calcium-binding protein (Yamaguchi and Yamamoto, 1975) and is found in the cytoplasm of liver cells (Takahashi and Yamaguchi, 1999). It is involved in the regulation of calcium metabolism and liver cell functions related to calcium (Akhter et al., 2006; Murata and Yamaguchi, 1998). In Table 2-B, it is observed as underexpressed. Interestingly, it was detected in a previous study that regucalcin gene expression was suppressed in rat HCC induced by a chemical carcinogen, 3'-methyl-4-dimethylaminoazobenzene (Makino and Yamaguchi, 1996). It is statistically significant ($P = 0.008$).

- Protein disulfide isomerase (PDI) is present in ER of liver cells (Akagi et al., 1988a). Also, it is found in the ER, Golgi, plasma membrane and secretory lumen of pancreatic exocrine cells (Akagi et al., 1988b). However, this association with the plasma membrane is due to a secretion of PDI in the case of pancreatic acinar cells. This is an exceptional situation and not the normal location for PDI. Under normal conditions it is associated only with the ER and is often used as a molecular marker for the ER (Khajavi et al., 2005). Therefore, it is an interesting protein since it might be also secreted in tumor cells. It has been described in association with major histocompatibility complex class I chain-related protein A at the surface of multiple myeloma cells (Jinushi et al., 2008) as well as the surface of solid tumor cells (Kaiser et al., 2007).

In conclusion, many of underexpressed proteins detected in tumor ER were proteins involved in general metabolism. As regard carbohydrate metabolism, there are

many related proteins, examples include, ketohexokinase (glycolysis) and fructose-1,6-biphosphatase (gluconeogenesis). This is in concordance with previous studies that indicated an altered carbohydrate metabolism in HCC (Lee et al., 2005), and in hematological cancers (Boag et al., 2006).

### A.1.1.3 Summary of comparison of our results with the results reported in the cancer literature

We observed many proteins that have been described as being differentially expressed in the cancer literature. Our results are different and new because we showed many of these proteins in a subcompartment of the cell, the ER and we have observed new proteins not previously described by the cancer literature in our study (described below).

### A.1.1.4 Differentially expressed phosphotyrosine proteins reported in cancer literature

The ER is a membrane-bound dynamic compartment that functions by interacting with cytoplasmic and luminal-soluble proteins. Among the proteins that interact with the ER membrane in a transient manner are tyrosine-phosphorylated proteins (Lavoie and Paiement, 2008). Table 3 shows examples of ER-associated proteins that have been identified as tyrosine-phosphorylated proteins (Rush et al., 2005). We also described the main function of each protein shown in this Table.

Overexpressed data are shown in a Table form (Table 3). Many proteins that are observed to be overexpressed in tumor ER have previously been described by Rush and colleagues (Rush et al., 2005). Some of these proteins are shown in Table 3. In the latter study, phosphoproteins were identified from human tumor cell lines by the detection of tyrosine-phosphorylated peptides via phosphoproteomic approach using mass spectrometry and immunoaffinity profiling.

**TABLE 3: Differentially expressed phosphotyrosine proteins reported in cancer literature** (Rush et al., 2005). The overexpression (ratio, R) [1] of the total peptides of smooth microsomes (SM) from control rat liver (Ctl) as regard to those from dissected liver tumor nodules (Nod) [SMNod/SMCtl] from 4 repeated mass spectrometry experiments (4N) were calculated.

| Protein (Rush et al., 2005) | Function [2] | Total peptides SMNod/SMCtl | Overexpression SMNod/SMCtl |
|---|---|---|---|
| Annexin II | Metabolism | 7.5/1 | 7.5 |
| Annexin I | Metabolism | 6/0 | 6/0 |
| Eukaryotic translation initiation factor 3, subunit 7 (zeta) (elF 3 subunit 7) | Protein synthesis | 3/0.5 | 6 |
| Poly A binding protein 1 (PABP 1) | Protein synthesis/ folding | 10.5/2.8 | 3.8 |
| Lung resistance related protein/major vault protein (LRP/MVP) | Detoxification | 34/10 | 3.4 |
| Y box protein 1 (YB 1) | Transcription | 3/1 | 3 |
| ATP citrate lyase | Lipid Metabolism | 16.8/8.5 | 2 |
| eukaryotic translation elongation factor 1 alpha 1 (eEF 1α) | Protein synthesis | 11.5/7.8 | 1.5 |
| Receptor of activated protein kinase C, 1 (RACK 1) | Signaling | 12.5/9.3 | 1.3 |

[1] $R \frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$

[2] Functional annotations were obtained primarily from Gilchrist and colleagues (2006).

Table 3, shows proteins we observed to be overexpressed and underexpressed in tumor ER in our mass spectrometry data. Many of these proteins are cytosolic (examples include annexin I, PABP 1, MVP, YB 1, ATP citrate lyase) (Gilchrist et al., 2006).

Since the identified proteins are involved in RNA, DNA and lipid metabolism, the observations prompt interest for the identification of tyrosine kinases that are responsible for tyrosine phosphorylation of these molecules and proteins. The finding of such tyrosine kinases might lead to potential drug targets for treatment of cancer.

### A.1.2 Differentially expressed proteins in ER from dissected liver tumor nodules: Our new findings

We observed above many proteins to be differentially expressed in tumor ER and many of these have previously been described in human HCC literature. In this section, we describe differentially expressed proteins of tumor ER that have not attracted much attention in the cancer literature. Such proteins could have cancer relevance or they may be relevant to carcinogenesis in general. Data for overexpressed and underxpressed proteins are shown in Table forms (Tables 4-A and 4-B respectively). We also described the main function of each protein shown in each Table.

#### A.1.2.1 Overexpressed proteins in ER from dissected liver tumor nodules

Overexpressed proteins are defined in our study, as the proteins with total number of peptides in the tumor ER were more than the total number of peptides in control ER fractions. Our results showed proteins that were overexpressed only in tumor ER. Many overexpressed proteins (Table 4-A) are involved in metabolism.

**TABLE 4-A: Most overexpressed proteins in ER from dissected liver tumor nodules.** The overexpression (ratio, R) [1] of the total peptides of smooth microsomes (SM) from control rat liver (Ctl) as regard to those from dissected liver tumor nodules (Nod) [SMNod/SMCtl] from 4 repeated mass spectrometry experiments (4N) were calculated.

| Protein | Function [3] | Total peptides SMNod/ SMCtl | Overexpression SMNod/ SMCtl |
|---|---|---|---|
| Annexin 1 | Lipid metabolism | 6/0 | - |
| Glucose-6-phosphate dehydrogenase | Carbohydrate metabolism | 3/0 | - |
| UDP-glucose 6-dehydrogenase | Metabolism | 21/0 | - |
| Cytokeratin 19 | Cytoskeleton | 6.8/0 | - |
| Uridine 5'-diphospho (UDP) glucuronosyltransferase 1A6 | Detoxification | 32.6/0.8 | 40 |
| rAFAR 1 | Detoxification | 17.8/0.8 | 23.6 |
| Glutathione S-transferase pi (GST pi) | Detoxification | 54.3/2.5 | 21.7 |
| Carbonic anhydrase 2 | Metabolism | 13.5/0.8 | 18 |
| Mannose 6-phosphate receptor | Transporter | 3.8/0.3 | 15 |
| 6-phosphogluconate dehydrogenase | Metabolism | 7.3/0.5 | 14.5 |
| Tripeptidyl peptidase II (TPP II) | MHC Class I | 17.4/1.3 | 13.8 |
| 15-Oxoprostaglandin 13-reductase | Metabolism | 23/1.8 | 13.1 |
| PREDICTED: similar to deoxyribose-phosphate aldolase-like | Carbohydrate metabolism | 2.5/0.3 | 10 |
| Transaldolase | Detoxification | 10.3/1.3 | 8.2 |
| Annexin II | Lipid metabolism | 7.5/1 | 7.5 |
| ATP-binding cassette sub-family C member 2 | Drug resistance | 6.3/1 | 6.3 |
| P55 | Unknown | 4.3/0.8 | 5.7 |
| hnRNP A2/B1 | mRNA metabolism | 9.7/1.7 | 5.5 |
| Ectonucleoside triphosphate diphosphohydrolase 5 (PCPH) | Nucleotide metabolism | 10.8/2 | 5.3 |
| Aldehyde dehydrogenase 1A1 | Metabolism | 10.5/2 | 5.3 |
| PREDICTED: similar to RNA helicase A | RNA metabolism | 6.3/1.5 | 5 |
| Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 3 Predicted | Protein degradation | 2/0.5 | 4 |
| Dynactin 1 | Cytoskeleton | 5.5/1.5 | 3.6 |
| Actin related protein 2/3, subunit2 | Cytoskeleton | 5.2/1.5 | 3.5 |
| PREDICTED: similar to GCN1 general control of amino-acid synthesis 1-like 1 | RNA metabolism | 22.3/6.5 | 3.4 |
| Glutathione S-transferase Ya-1 | Detoxification | 20/6 | 3.2 |
| Annexin III (Lipocortin III) | Lipid metabolism | 2.7/0.9 | 3 |
| Liver carboxylesterase 4 precursor | Detoxification | 49.8/18.3 | 2.7 |
| Transketolase | Metabolism | 29.5/12.2 | 2.4 |
| hnRNP K | mRNA metabolism | 14.5/6.3 | 2.3 |
| Microsomal epoxide hydrolase | Detoxification | 37.5/16.25 | 2.3 |
| ATP-citrate (Pro-S-)-lyase | Lipid metabolism | 21.8/9.7 | 2.2 |
| hnRNP M | mRNA metabolism | 1/0.5 | 2 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (DEAD box protein 1) | mRNA metabolism | 8.2/4.2 | 1.9 |
| Sec31 like-1 protein | Membrane traffic | 31.3/19 | 1.6 |
| 14-3-3 protein isoform zeta rat (fragment) | Signaling | 7.4/5.1 | 1.4 |

[1] R $\frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$

[2] Functional annotations were obtained primarily from Gilchrist and colleagues (2006).

Table 4-A, shows proteins we observed to be overexpressed in ER from dissected tumor nodules. We have chosen to discuss details of the following proteins for two reasons. One they are highly overexpressed in our study and two they have been reported by others either to play an important role in ER fraction or to be differentially expressed in cancer. Proteins are statistically significant when *P-value* is $\leq 0.05$ (this means that the total number of peptides for a given protein was determined to be statistically different when compared between tumor and control membranes. A statistical difference was considered significant when the comparison of the means of the total peptides gave a *P-value* is $\leq 0.05$ by paired-samples T tests). Overexpressed proteins of note are as follows:

- Uridine 5'-diphospho (UDP) glucuronosyltransferase 1A6 is an enzyme that is highly expressed in the liver and is involved in detoxification. It catalyses the glucuronidation of many toxic compounds forming glucuronide conjugates, the latter are more water-soluble than the initial compounds (Auyeung et al., 2003). It is highly overexpressed protein in this Table (40-fold increase) and it is statistically significant (*P = 0.02*).

- Rat aflatoxin B1 aldehyde reductase member 1 (rAFAR 1) is a well-known cytsolic protein. It belongs to the aldo-keto reductase superfamily and is involved in aflatoxin detoxification (Knight et al., 1999; Praml et al., 2003; Zeindl-Eberhart et al., 2001). Rat aflatoxin B1 aldehyde reductase member 1 is among the most overexpressed proteins in Table 4-A (24-fold increase). This result is consistent with study of Ellis and colleague (Ellis et al., 1993) that showed around 20-fold increase after xenobiotics treatment. It is statistically significant (*P = 0.02*).

- Glutathione S-transferase pi (GST pi) is one of the cytosolic proteins belonging to the GST family and is involved in detoxification (Aliya et al., 2003; Zeindl-Eberhart et al., 2001). It was observed to be one of the most overexpressed proteins in tumor ER (22-fold increase) over control liver ER. It is statistically significant ($P = 0.007$).

- Ectonucleoside triphosphate diphosphohydrolase 5 (PCPH) is involved in nucleotide metabolism. The cancer process may be associated with alterations in the PCPH proteins (Rouzaut et al., 2001). It is a secretory protein in human testicular tumor (Regadera et al., 2006). On the other hand, its immunohistochemical expression was abolished in malignant evolution of precancerous lesions of the larynx in human, hence suggesting its use as a reliable marker for the malignant progress in larynx tumor (Blanquez et al., 2002). It is statistically significant ($P = 0.01$).

- Transketolase is involved in the non-oxidative branch of pentose phosphate pathway (Boren et al., 2006). It was overexpressed in our data, this was also observed in a previous proteomic study for pancreatic tumor in human (Gronborg et al., 2006). It is statistically significant ($P = 0.007$).

- Glucose-6-phosphate dehydrogenase is involved in carbohydrate (glucose) metabolism (Sturman, 1967). It is a regulatory enzyme of the pentose phosphate pathway (oxidative branch) (Wagner et al., 1978). In a study it was used as tool to help in diagnosis of pancreatic adenocarcinoma (Van Driel and Van Noorden, 1999). In addition, it was considered a potential urinary marker for human bladder tumor (Lin et al., 2006). It is statistically significant ($P = 0.04$).

- Cytokeratin 19 is a component of the intermediate filament cytoskeleton in the cytoplasm of epithelial cells (Hofler et al., 1984; Lazarides, 1982). It can be used to evaluate pathological progression in HCC and as a useful marker for predicting tumor metastases and a therapeutic target for the treatment of HCC patients with metastases (Ding et al., 2004). It is statistically significant ($P = 0.04$).

In conclusion, many of overexpressed proteins detected in ER from dissected tumor nodules were proteins involved in metabolism and detoxification. As regard carbohydrate metabolism, there are PPP-related proteins, examples include, transketolase (non-oxidative branch) and Glucose-6-phosphate dehydrogenase (oxidative branch). While for detoxification-related proteins, examples include rAFAR 1 and GST pi. They are phase I (Jin and Penning, 2007) and phase II (Aliya et al., 2003) drug-metabolizing enzymes respectively. Aflatoxin detoxification involves targets aflatoxin gene activation. This result is expected because of aflatoxin poisoning. Other important proteins to mention are the Heterogeneous nuclear ribonucleoproteins A2/B1, K and M are related to RNA metabolism. The RNP family protein members detected in ER may be associated with membrane-bound polysomes and modulate translation of specific proteins relevant to protein synthesis, proteins mediating targeting, co-translational translocation, and processing of nascent polypeptide chains (Lavoie and Paiement, 2008).

#### A.1.2.1.1 Proteasome enzymes

Many proteins related to the proteasomes were observed as being overexpressed in tumor ER. The proteolytic activity is achieved by the action of proteasome/ubiquitination system. Initially, multiple ubiquitin molecules tag the protein needed to be degraded; this is followed by degradation of the ubiquitinated proteins by the multicatalytic proteinase called the 26S proteasome complex with release of free ubquitin to be recycled and short peptides products (Hochstrasser, 1996). Proteomics analysis of ER membranes revealed many of the proteasome subunits to be mostly localised in the smooth microsomes (high in SMNod and SMCtl but low in RMNod, Figure 5). This data is consistent with a previous report showing that proteasomes were associated with the smooth endoplasmic reticulum and that they were almost absent from the rough endoplasmic reticulum (Palmer et al., 1996). In addition, this data is consistent with the proteomic study of Gilchrist and colleagues (Gilchrist et al., 2006) that showed proteasome subunits only in smooth ER. Other recent study on HBV-induced HCC in mice is in accordance with our data, indicating the possibility that the increased

proteasomes could enhance the altered degradation of cellular regulators that control the proliferation and apoptosis of normal and tumor cells (Cui et al., 2006).

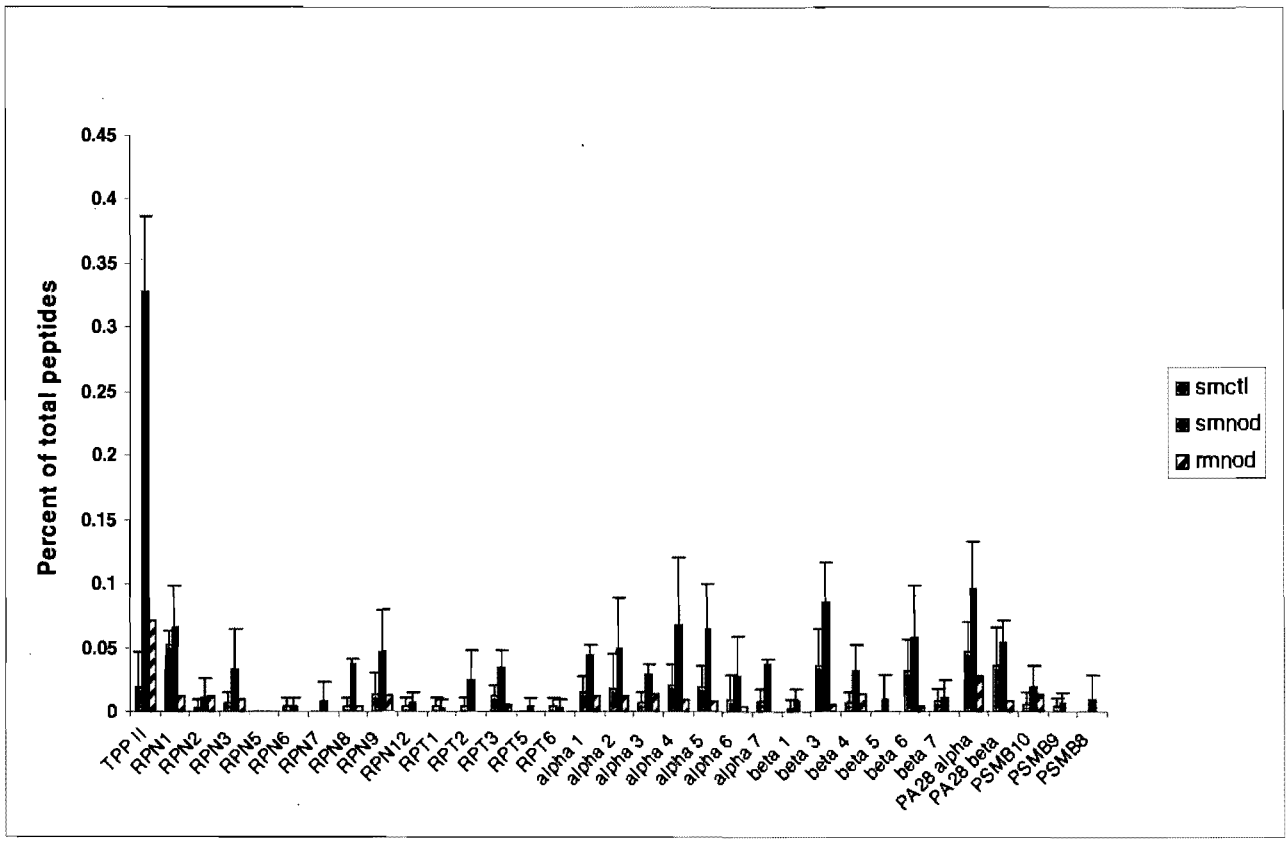
- Tripeptidyl peptidase II (TPP II) which is observed as overexpressed in Table 4-A and Figure 5, is an enzyme involved in the proteolytic degradation of peptides provided by the proteasome (Tomkinson and Lindas, 2005). The latter is involved in the generation of MHC class I ligands (Marcilla et al., 2008) TPP II enzyme may act as a downstream of the proteasome, by processing most proteasomal degradation products before they enter the ER (Reits et al., 2004). The TPP II is expressed in our data as 14-fold increase and include data for TPP II because of its role in generation of MHC class I peptides. Figure 5 shows all subunits of the proteasome peptides for the various subunits were detected in our mass spectrometry data for SMCtl, SMNod and RMNod membranes.

**FIGURE 5: Peptides of proteins of proteasome in ER of dissected liver tumor nodules and in ER of control rat liver.** Different subunits [1] of the proteasome in three different ER subfractions smooth microsomes from control rat liver (smctl), smooth microsomes from dissected liver tumor nodules (smnod) and rough microsomes from dissected tumor nodules (rmnod) are shown on the X-axis. The percent of total peptides of each of them is shown on the Y-axis [2]. Percent of total peptide was determined by adding the sum of peptides for each protein from three experimental fractions. The mean was then expressed as a percent of the total peptides of all the proteins detected in the same three experimental fractions. Mean number of peptides are shown with standard deviations. Mean number of peptides ranged from 0 (example PSMB 8, smctl) to 14.5 (example TPP II, smnod).

---

[1] TPP II (tripeptidyl-peptidase 2), RPN (proteasome 26S subunit, non-ATPase 1, 2, 3, 5, 6, 8, 9 and 12 subunits); RPT (proteasome 26S subunit, ATPase 1, 2, 3, 5 and 6); alpha (proteasome subunit, alpha type 1, 2, 3, 4, 5, 6 and 7), beta (proteasome subunit, beta type 1, 3, 4, 5, 6 and 7), PA 28 (proteasome 28 subunit, alpha and beta) and PSMB (proteasome subunit, beta type 10, 9 and 8).

[2] The values on the Y-axis represent the number of unique peptides found for a certain protein, divided by the number of all peptides detected in the ER subfraction.


Percent of total peptides
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
smctl
smnod
rmnod
TPP II
RPN1
RPN2
RPN3
RPN5
RPN6
RPN7
RPN8
RPN9
RPN12
RPT1
RPT2
RPT3
RPT5
RPT6
alpha 1
alpha 2
alpha 3
alpha 4
alpha 5
alpha 6
alpha 7
beta 1
beta 3
beta 4
beta 5
beta 6
beta 7
PA28 alpha
PA28 beta
PSMB10
PSMB9
PSMB8

Many of proteasome subunits were overexpressed in the SMNod. Proteasome subunits are statistically significant when *P-value* is $\leq 0.05$ (this means that the total number of peptides for a given protein was determined to be statistically different when compared between tumor and control membranes. A statistical difference was considered significant when the comparison of the means of the total peptides gave a *P-value* is $\leq$ 0.05 by paired-samples T tests). These include TPP II (statistically significant, $P = 0.02$), RPN 8 (statistically significant, $P = 0.4$), RPT 2 (statistically insignificant, $P = 1$), alpha 7 (statistically insignificant, $P = 0.2$), beta 4 (statistically insignificant, $P = 0.2$) and PA 28 alpha (statistically insignificant, $P = 0.2$). The latter together with PA 28 beta are subunits of proteasome involved in production of peptides specifically involved in MHC class I dependent immumosurveillance (Kloetzel, 2004; Kloetzel and Ossendorp, 2004).

In conclusion, newly synthesized unfolded and unassembled proteins become substrates for the ER-associated degradation process. This well-regulated process causes retention of defective proteins in the ER. Finally, these proteins become exported in the cytoplasm and targeted for polyubiquitination and degradation by the 26S proteasome complex (Meusser et al., 2005; Romisch, 2005). Moreover, different subunits of proteasomes with localisation mostly to SM membrane were detected; this probably reflects differences in the function of proteasomes in distinct cell compartments (Palmer et al., 1996).

### A.1.2.2 Underexpressed proteins in ER from dissected liver tumor nodules

Underexpressed proteins are defined in our study, as the proteins with total number of peptides in the tumor ER were less than the total number of peptides in control ER fractions. Our results showed proteins that were underexpressed only in tumor ER. Many underexpressed proteins (Table 4-B) are involved in detoxification.

**TABLE 4-B: Most underexpressed proteins in ER from dissected liver tumor nodules.** This Table is based on the same idea as (Table 4-A). The underexpression (ratio, R) [1] of the total peptides of smooth microsomes (SM) from control rat liver (Ctl) as regard to those from dissected liver tumor nodules (Nod) [SMNod/SMCtl] from 4 repeated mass spectrometry experiments (4N) were calculated.

| Protein | Function [2] | Total peptides SMNod/ SMCtl | Underexpression SMNod/ SMCtl |
|---|---|---|---|
| Solute carrier organic anion transporter family member 1A4 | Transporter | 0/11.5 | 0 |
| Cytochrome p450 2C7 | Detoxification | 0.5/15.4 | 0.03 |
| Carnitine palmitoyltransferase 1, liver | Lipid Metabolism (Mito) | 0.5/5 | 0.1 |
| | | | |
| Choline dehydrogenase | Metabolism | 0.8/3 | 0.3 |
| Glycogen synthase 2 | Metabolism | 1/3.8 | 0.3 |
| Aldehyde dehydrogenase 9A1 | Metabolism | 0.5/4 | 0.1 |
| Myosin heavy chain | Cytoskeleton | 1.3/12.5 | 0.1 |
| Estrone sulfotransferase | Metabolism | 0.5/9.2 | 0.1 |
| Cytochrome p450 3A18 | Detoxification | 0.5/6.2 | 0.1 |
| Cytochrome p450 2A2 | Detoxification | 1.8/12 | 0.1 |
| Carbonic anhydrase III | Metabolism | 3.8/25 | 0.2 |
| Adenosine kinase | Metabolism | 0.8/4.5 | 0.2 |
| Cytochrome p450 2C6 | Detoxification | 4.5/18.8 | 0.2 |
| Cytochrome p450 2C11 | Detoxification | 10.3/52.4 | 0.2 |
| Argininosuccinate synthase | Metabolism | 5/31 | 0.2 |
| Alpha-2u globulin | Secretory protein | 6.5/33.7 | 0.2 |
| Cytochrome p450 2D2 | Detoxification | 10.1/29.2 | 0.3 |
| Glucose 1-dehydrogenase, microsomal | Carbohydrate metabolism | 4.8/13.5 | 0.4 |
| Fumarylacetoacetase | Metabolism | 6/13.8 | 0.4 |
| Liver glycogen phosphorylase | Metabolism | 22.3/28.8 | 0.8 |

[1] $R \frac{SMNod}{SMCtl} = \frac{N1+N2+N3+N4}{N1+N2+N3+N4} = \frac{4N}{4N}$

[2] Functional annotations were obtained primarily from Gilchrist and colleagues (2006).

Table 4-B, shows proteins we observed to be underexpressed in ER from dissected tumor nodules. We have chosen to discuss details of the following proteins for two reasons. One they are highly underexpressed in our study and two they have been reported by others either to play an important role in ER fraction or to be differentially expressed in cancer. Proteins are statistically significant when *P-value* is $\leq 0.05$ (this means that the total number of peptides for a given protein was determined to be statistically different when compared between tumor and control membranes. A statistical difference was considered significant when the comparison of the means of the total peptides gave a *P-value* is $\leq 0.05$ by paired-samples T tests). Underexpressed proteins of note are as follows:

- Six members of the cytochrome p450 (CYP 450) family have been detected in our quadriplet mass spectrometry data as underexpressed in the ER tumor over the ER control. The cytochrome p450 protein family is involved in detoxification (Guengerich et al., 1996). CYP 450 have also been reported in numerous cancer literature studies as underexpressed, example include CYP 450 2E1 in human HCC (Kinoshita and Miyata, 2002). According to our mass spectrometry data this CYP 450 sub-family (that is not shown in Table 4-B) was also underexpressed (total number of peptides in SMNod were 32 and 62 in SMCtl). The downregulation of this detoxification enzyme system might increase the effects of aflatoxin poisoning. All the six-cytochrome p450 family members are statistically significant as follows: 2C7 ($P = 0.03$), 3A18 ($P = 0.059$), 2A2 ($P = 0.01$), 2C6 ($P = 0.03$), 2C11 ($P = 0.004$) and 2D2 ($P = 0.01$).

- Glycogen synthase 2 (Gys 2) is involved in metabolism being the rate-limiting enzyme for glycogen synthesis in liver and adipose tissue. Under normal conditions, glycogen storage is mainly in liver, muscle, and adipose tissue. Liver glycogen serves to maintain blood glucose levels between meals, while skeletal muscle glycogen is used to fuel muscle contractions (Cheng et al., 2006; Mandard et al., 2007). On the other hand, adipose tissue glycogen serves as a source of glycerol 3-phosphate, which is required for esterification of fatty acids into triglycerides (Antwi et al., 1988). In Table 4-B it is underpexressed. In one of the interesting studies, Gys 2 gene was reported as

overexpressed in HCC associated with hepatitis C virus (Iizuka et al., 2002). On the other hand, the gene for Gys 2 was underexpressed in human cervical cancer associated with human papilloma virus serotype 16 (Wong et al., 2006).

- Liver glycogen phosphorylase is involved in metabolism, as it serves in glycogen breakdown leading to production of free glucose 1-phosphate to be used for glycolysis and various synthetic functions. The enzyme is found mainly in three forms, named liver, brain and muscle according to their corresponding predominant expression (Chang et al., 1998; Cheng et al., 2006). In Table 4-A, the liver form was underexpressed and statistically significant ($P = 0.05$).

- Estrone sulfotransferase (or estrogen sulfotransferase) is involved in steroid metabolism, being a sex steroid hormone. It sulfates hydroxysteroids by transferring the sulphate group from phosphoadenosine-phosphosulfate (Demyan et al., 1992). Normally, estrogen is a sex steroid hormone; it is mainly a female hormone since it is synthesized in the ovary and plays a critical role in female reproduction. However, this hormone is also important in male reproduction organs and other tissues including bone, liver, the central nervous system, and the vascular system. Estrogen exerts its physiological role through oestrogen receptors. The latter are widely expressed in a variety of tissues (Miki et al., 2002). This enzyme was underexpressed in Table 4-B, in a recent human bone cancer study it was overexpressed (Svoboda et al., 2007). It is statistically significant ($P = 0.04$).

In conclusion, many of the underexpressed proteins in ER from dissected tumor nodules are involved in various metabolic pathways. Examples include enzymes involved in glycogen metabolism, glycogen synthase 2 and glycogen phosphorylase and proteins involved in steroid metabolism, estrone sulfotransferase. Also, we detected the underexpression of many protein members of the cytochrome p450 family.

### A.1.3 Proteins showing no significant difference in concentration between control ER and tumor ER

Many proteins were detected in significant amounts in control ER and tumor ER based on peptide counting. Here we draw attention to two categories of proteins, proteins involved in folding and proteins involved in histocompatibility antigen presentation because these proteins were observed in equal amounts in control ER and in tumor ER.

#### A.1.3.1 Proteins involved in folding

The endoplasmic reticulum is the site where the majority of secreted proteins and membrane proteins are folded (Hendershot, 2004). Figure 6 shows the relative amounts of peptides of numerous folding proteins detected in control and tumor ER.

**FIGURE 6: Peptides of folding proteins in ER of dissected liver tumor nodules and in ER of control rat liver.** Different proteins [1] belonging to the folding protein family in the three different ER subfractions which were described before [2] are shown on the X-axis. The percent of total peptides of each of them is shown on the Y-axis. The percent of total peptide and mean number of peptides were determined as described before [2]. Mean number of peptides ranged from 0 (example DnaJ C memb 13, smctl) to 18.3 (example PDI, rmnod).

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[1] Bip/GRP 78 (immunoglobulin heavy chain-binding protein/78 kDa glucose-regulated protein precursor), gp96, PDI (protein disulfide isomerase), PDI A3 (protein disulfide isomerase A3), Orp 150 (hypoxia up-regulated protein 1 precursor (150 kDa oxygen-regulated protein), PDI A4 (protein disulfide isomerase A4), calreticulin, calnexin, cyclophilin A, regucalcin, PDI A5 (protein disulfide isomerase A5), Hsp 90 (heat shock protein 90), PDI A6 (protein disulfide isomerase A6), Hsp 85 (heat shock protein 85), tapasin, ERp 45 (endoplasmic reticulum protein 45), ERp 20, Hsp 70 (heat shock protein 70), ERp 19 (endoplasmic reticulum protein 19), ERp 44 (endoplasmic reticulum protein 44), DnaJ C memb 13 (DnaJ (Hsp40) homolog, subfamily C, member 13) and Hsp 47 (heat shock protein 47).

[2] As described before in Figure 5.

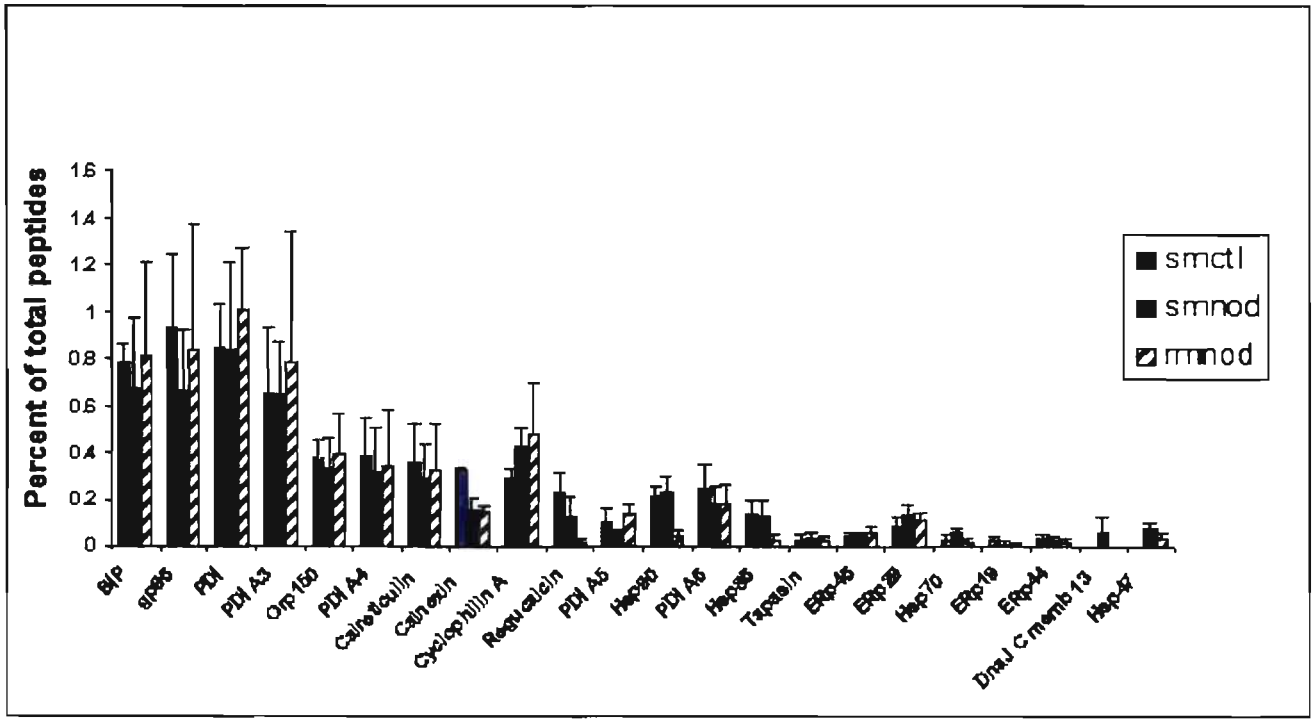

Percent of total peptides
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
smctl
smnod
rmnod
BiP
gp96
PDI
PDI A3
Orp150
PDI A4
Calreticulin
Calnexin
Cyclophilin A
Reticulocalbin
PDI A5
Hsp90
PDI A6
Hsp60
Taxpolin
ERp46
ERp72
Hsp70
ERp19
ERp44
DnaJ C membr 13
Hsp47

Figure 6 shows that the folding proteins with the highest number of peptides are Bip, gp 96, PDI and PDI A3. Bip also known as immunoglobulin heavy chain-binding protein is shown in Figure 6 with mean number of peptides 18 in tumor ER (SMNod) and 18.8 in control ER (SMCtl). Of particular interest, Figure 6 reveal equal numbers of peptides for the majority of the proteins in control ER and tumor ER. Folding proteins of note are as follows:

- Immunoglobulin heavy chain-binding protein/78 kDa glucose-regulated protein precursor (Bip/GRP 78) is an ER resident chaperone protein (Bertolotti et al., 2000) and it is a member of the HSP 70 family of heat shock proteins that act as chaperones (Awad et al., 2008). It interferes to correct misfolded proteins, which are present in the lumen of ER (Hendershot, 2004). It is equally expressed in control rat liver and dissected liver tumor nodules fractions. This is in contrast to previous studies in literature that stated that Bip is overexpressed in human HCC (Sun et al., 2007), and this could be explained by the fact that their study was done on tissues whereas ours on ER-derived fractions.

- Of note was the fact that calnexin was an exception amongst the folding proteins. Unlike other folding proteins, calnexin was underexpressed in tumor ER (Figure 6).

### A.1.3.2 Proteins involved in histocompatibility antigen presentation

Certain proteins are part of the major histocompatibly class I (MHC class I) peptide loading complex (PLC). This complex is made up of many proteins that include MHC class I molecules, beta 2 microglobulin, the chaperone calreticulin, the oxidoreductases ERp57, and protein disulfide isomerase, the class I-specific accessory molecule tapasin and the peptide transporter TAP (Elliott, 2006). This complex is involved in the establishment of proper conformation of MHC class I molecules for peptide loading in the ER and forms part of the antigen presenting machinery which is transported to the plasma membrane where antigenic peptides become available for immune surveillance (Hammer et al., 2007). Most of proteins of the loading complex

were detected in control ER and tumor ER and the relative numbers of peptides for these proteins are shown in Figure 7.

**FIGURE 7: Proteins in the major histocompatibility class I (MHC class I) peptide loading complex (PLC).** Different proteins [1] belonging to the PLC in the three different ER subfractions which were described before [2] are shown on the X-axis. The percent of total peptides of each of them is shown on the Y-axis. Percent of total peptide and mean number of peptides were determined as described before [2]. Mean number of peptides ranged from 0.3 (example RT1BI alpha chain, smnod) to 18.3 (example PDI, rmnod).

---

[1] ERp 57 (endoplasmic reticulum protein 57), calreticulin, PDI (protein disulfide isomerase), ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing), β2 micorglobulin (beta 2 microglobulin), tapasin (tap-binding protein), TPP II (tripeptidyl-peptidase 2), TAP 2 (transporter associated with antigen processing 2), TAP 1 (transporter associated with antigen processing 1), rat MHC class I (rat major histocompatibly complex class I) and RT1BI alpha chain.

[2] As described before in Figure 5.

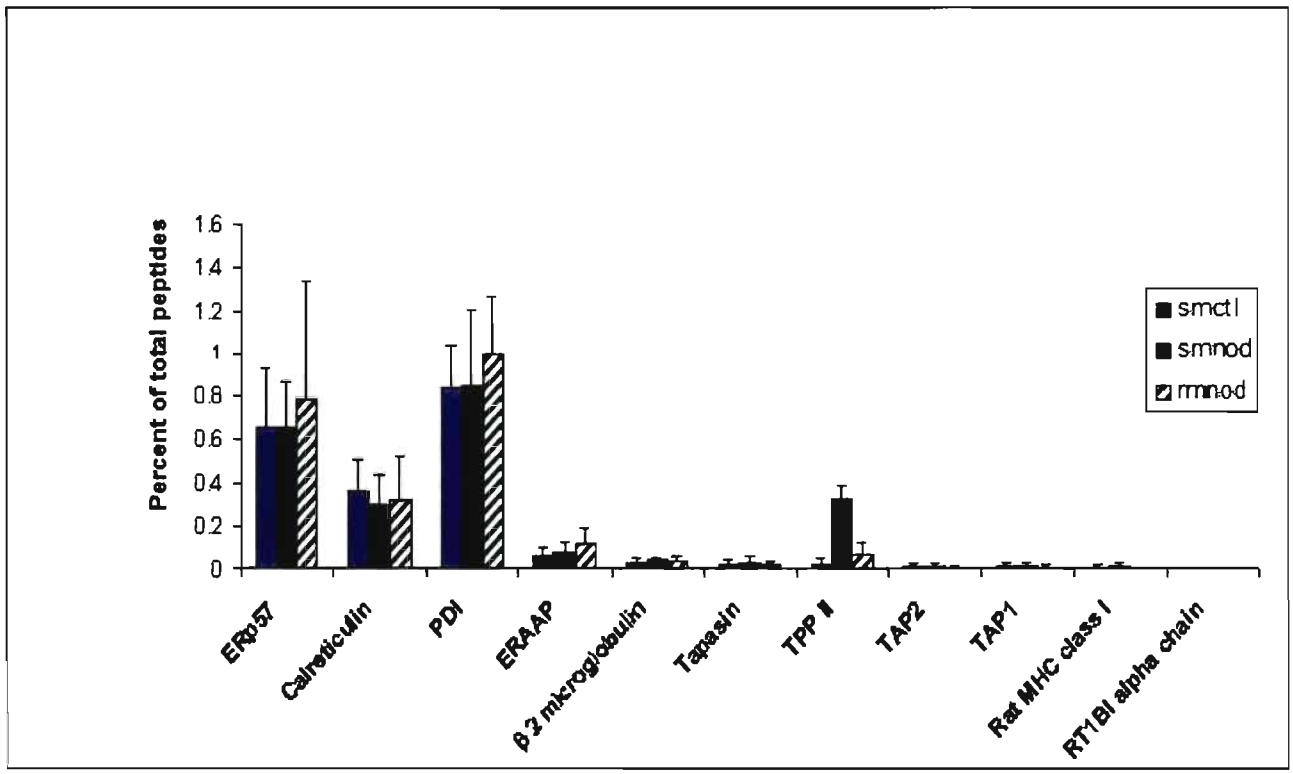

Percent of total peptides
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
smctl
smmod
rmmod
ERp57
Calreticulin
PDI
ERAAP
β 2 microglobulin
Tapasin
TPP II
TAP2
TAP1
Rat MHC class I
RT1.Bl alpha chain

Figure 7 shows that proteins of the PLC with the highest concentration of peptides are ERp 57, calreticulin, PDI and TPP II. Protein disulfide isomerase is shown in Figure 7 with mean number of peptides 8.8 in tumor ER (SMNod) and 11.8 in control ER (SMCtl). With exception of TPP II, all proteins were detected with similar amount of peptides in control ER and tumor ER. This is an interesting finding because in a previous report in literature has indicated underexpression of proteins of PLC in cancer (Dissemond et al., 2004) and because underexpression of these components has previously been implicated in escape of immune surveillance. We notice no significant expression change in the three experimental fractions (Figure 7).

## A.2 Unknown proteins

### A.2.1 Novel proteins detected in ER

Both known and unknown proteins were detected by our mass spectrometry study. We have chosen to call the unknown proteins novel endoplasmic reticulum associated protein (NERA). We detected many novel proteins 20 of them are shown in Figure 8.

**FIGURE 8: Novel endoplasmic reticulum associated proteins (NERA).** The number of peptides is shown for 20 novel proteins [1] in the three different ER subfractions, which were described before [2]. The percent of total peptides of each of them is shown on the Y-axis. Percent of total peptide and mean number of peptides were determined as described before [2]. Mean number of peptides ranged from 3.8 (example NERA 5, smctl) 16.8 (example NERA 3, rmnod). Each novel protein was also characterized according to T, P and A domains [3].

---

[1] Nera 3 (PREDICTED: similar to KIAA0372), Nera 16 (Leucine-rich repeat-containing protein 59 Protein p34), Nera 4 (PREDICTED: similar to mKIAA0183 protein), Nera 1 (PREDICTED: similar to 5730439E10Rik protein), Nera 15 (PREDICTED: similar to RIKEN cDNA C230096C10), Nera 2 (Protein FAM98A), Nera 7 (Protein KIAA0152 homolog precursor), Nera 6 (WD repeat protein 61), Nera 5 (LRRGT00164), Nera 14 (PREDICTED: similar to RIKEN cDNA 5730596K20), Nera 17 (PREDICTED: hypothetical protein XP_217094), Nera 20 (Protein C10orf58 homolog precursor), Nera 19 (Hypothetical protein Mettl7b), Nera 8 (PREDICTED: similar to KIAA1033 protein), Nera 9 (PREDICTED: similar to Dendritic cell protein GA17), Nera 10 (PREDICTED: similar to Hypothetical protein MGC31278), Nera 13 (PREDICTED: similar to RIKEN cDNA 2310008M10), Nera 11 (PREDICTED: similar to protein HT031 homolog [Rattus norvegicus]), Nera 12 (Zinc finger protein 294 (Zfp-294) [Mus musculus]) and Nera 18 (PREDICTED: similar to 2610030H06Rik protein).

[2] As described before in Figure 5.

[3] T (TMD, transmembrane domain), P (signal peptide) and A (signal anchor) domains from database (TMD: http://www.cbs.dtu.dk/services/TMHMM/, signal P and signal anchor: http://www.cbs.dtu.dk/services/SignalP/). $T_1$ = 1 transmembrane domain, $T_2$ = 2 transmembrane domains, $T_3$ = 3 transmembrane domains and $T_4$ = 4 transmembrane domains.

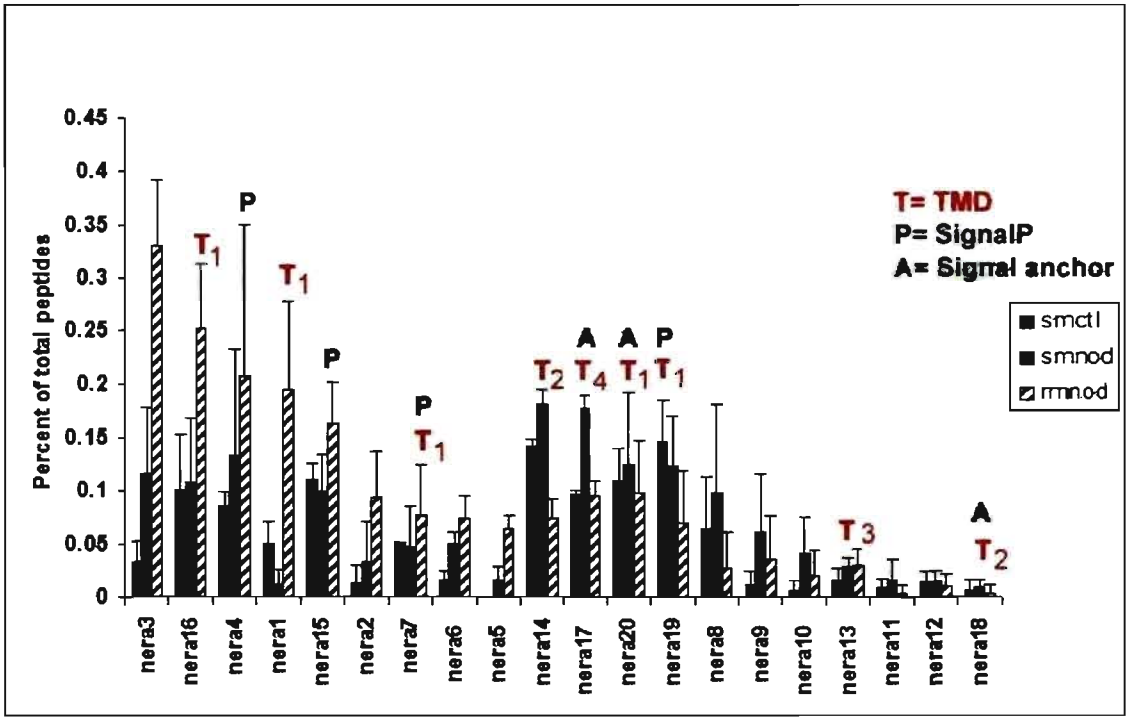

Percent of total peptides
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
T= TMD
P= SignalP
A= Signal anchor
smctl
smnod
rmnod
nera3
nera16
nera4
nera1
nera15
nera2
nera7
nera6
nera5
nera14
nera17
nera20
nera19
nera8
nera9
nera10
nera13
nera11
nera12
nera18

Figure 8, shows novel proteins we observed with differential expression in tumor ER. Prediction algorithms identified 9 putative transmembrane proteins (Nera 16, 1, 7, 14, 17, 20, 19, 13 and 18), 4 putative secretory proteins (Nera 4, 15, 7 and 19) and 3 proteins with signal anchor (Nera 17, 18 and 20). NERA proteins shown on the left of the Figure (Nera 3, 16, 4, 1, 15, 2, 7, 6 and 5) are thought to be enriched in rough microsomes because they were observed in high concentrations in rough microsomes from tumor nodules (see peptide counts for RMNod). Nera proteins shown on the right of the Figure (Nera 14, 17, 20, 19, 8, 9, 10, 13, 11, 12 and 18) are thought to be enriched in smooth microsomes (see peptide counts for SMCtl and SMNod).

Other unknown proteins are detected, among them the homologue of KIAA0196 (strumpellin) that is observed on our data as overexpressed (with mean number of peptides 1 in tumor ER (SMNod) and 0.3 in control ER (SMCtl)). The protein is involved in spinal cord disease and paraplegia in human (Valdmanis et al., 2007).

NERA 14 revealed a significant difference in expression when peptide numbers were compared between smooth microsomes from control and smooth microsomes from tumor nodules. Therefore, this protein is a potential tumor marker.

## PART B: Validation of mass spectrometry (MS) results

Mass spectrometry is a bioinformatic tool that yields probability data (Tibshirani et al., 2004). Such data should be validated using more direct techniques for detecting proteins. Therefore, in this study we used quantitative immunoblot analysis to confirm the MS data (Qi et al., 2008).

### B.1 Known proteins

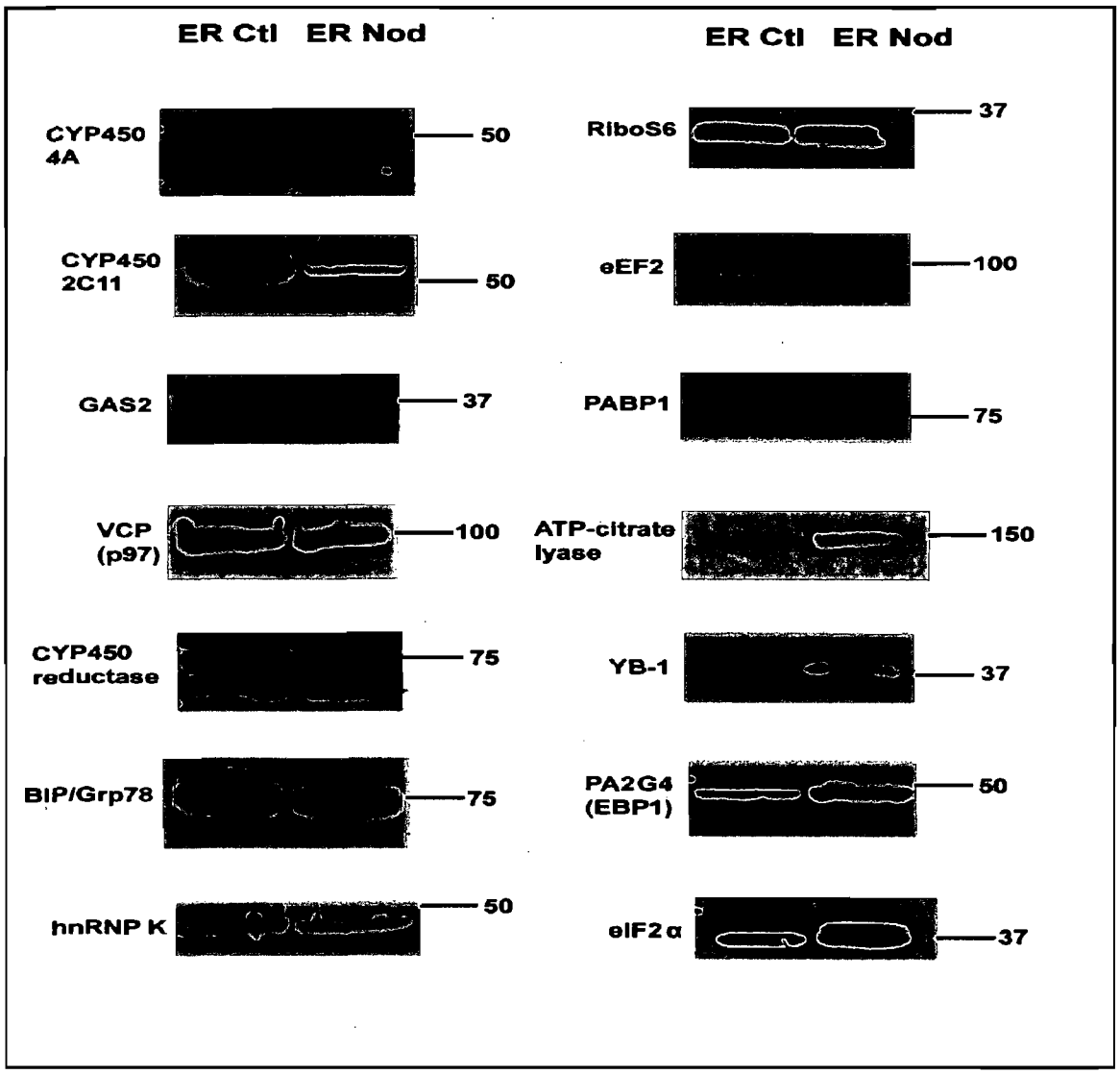
Immunoblot (Western blot) experiments were carried out in a way to provide a quantitative validation of cancer relevant proteins detected by mass spectrometry.

#### B.1.1 Endoplasmic reticulum (control and tumor) fractions

##### B.1.1.1 Validation of mass spectrometry results using antibodies directed against cancer related proteins

Immunoblot experiments were performed using specific antibodies relevant to cancer proteins. The choice of antibodies was based on the availability of high quality antibodies (with good specificity and high affinity) to proteins shown by the scientific literature to be important in human cancer. Figure 9 shows that 7 of 14 proteins (PA2G4, eEF 2, eIF 2α, YB 1, hnRNP K, ATP citrate lyase and PABP 1) are overexpressed while only 4 of 14 proteins (CYP450 4A, CYP450 2C11, CYP450 reductase and GAS 2) are underexpressed in nodular fraction. However the rest of the proteins which are 3 of 14 (VCP, Bip and Ribo S6) are almost equally expressed in both fractions (nodular and control).

**FIGURE 9: Immunoblots for known proteins** [1]. Immunoblots of selected proteins found in smooth microsomes from control rat liver (Ctl) and in smooth microsomes from dissected liver tumor nodules (Nod). Equal protein aliquots (40 μg) were loaded onto mini gels for control and tumor microsomes. Following electrophoresis separation proteins were transferred onto nitrocellulose membranes (Bio Rad) and revealed by immunoblots with the indicated antibodies. All antibodies except those against Gas 2 (home prepared) and rAFAR 1 (generous gift of Dr John Hayes, Dundee Scotland) were from commercial sources (identified in the Materials and Methods, Table 1).




[1] Genevieve Thibault did these experiments.

**B.1.1.1.1 Overexpressed tumor ER proteins**

- Proliferation-associated protein 2G4/ErbB3 binding protein 1 (PA2G4/EBP 1) is a cell cycle protein (Yoo et al., 2000). PA2G4 is localized to the cytoplasm and to the nucleolus. It is a part of ribonucleoprotein complexes. It plays a role in cell proliferation (Squatrito et al., 2004).

- Eukaryotic translation elongation factor 2 (eEF 2) is involved in mRNA metabolism (Taylor et al., 2007). It is overexpressed in HCC (Li et al., 2008). Under hypoxic conditions, the enzymatic activity of this protein can be suppressed (Liu et al., 2006).

- Eukaryotic translation initiation factor 2, subunit 1 alpha (eIF 2α) is involved in mRNA metabolism (Miyamoto et al., 1996) as eEF 2. Anoxic conditions, which characterise tumor, result in disruption of eIF 2α phosphorylation with subsequent eIF 2α dependent inhibition of overall mRNA translation, differential gene expression, hypoxia tolerance and tumor growth (Koritzinsky et al., 2007; Liu et al., 2006).

- Y box protein 1 (YB 1) is a transcription factor and marker for breast cancer. It correlates with high aggressiveness of the tumor and with chemoresistance (Fujita et al., 2005).

- Poly A binding protein 1 (PABP 1) is involved in RNA metabolism as eEF 2 and eIF 2α. It binds the poly (A) tail of mRNA and is involved in activating the stability and translation of mRNAs by protecting poly (A) tail (Penalva et al., 2004) from exonucleases (Bernstein et al., 1989; Gallie, 1998). So like that, PABP 1 is involved in protein synthesis. In addition, it plays a role in cell cycle regulation (Penalva et al., 2004) and it is tyrosine phosphorylated (Table 4).

- Heterogeneous nuclear ribonucleoprotein K (hnRNP K) was also observed as overexpressed by immunoblot analysis. This is a tumor marker and the significance of this protein has already been discussed (see text for Table 2-A).

- Adenosine triphosphate (ATP) citrate lyase plays an important role in de novo lipogenesis pathways. It is an enzyme that is critical for the conversion of glucose to cytosolic acetyl CoA (the later is a basic unit for all endogenous fatty acid and sterol synthesis) and therefore for glucose-dependent lipogenesis in growing cells. It is also required to maintain rapid cell proliferation, suggesting that glucose-dependent lipogenesis is an important component of cell growth (Bauer et al., 2005).

**B.1.1.1.2 Underexpressed tumor ER proteins**

- Cytochrome p450 (CYP 450) members CYP 4A and CYP 2C11 and reductase were underexpressed in the ER tumor membranes. The superfamily cytochrome p450 is involved in detoxification (Anzenbacher and Anzenbacherova, 2001). Antibody anti-CYP450 4A recognizes both CYP450 4A1 and 4A2. However, the mass spectrometry data used was that related to CYP450 4A1. The CYP 450 reductase, which is otherwise named as NADPH--cytochrome P450 reductase is not really a member of the CYP 450 superfamily, however it works with CYPs members, being considered as a redox partner of human liver CYPs (Petushkova et al., 2006).

- Growth arrest-specific protein 2 (GAS 2) protein is also underexpressed in tumor ER. This protein is involved in p53 dependent cellular apoptosis following DNA damage (Benetti et al., 2001). Therefore, it would be expected that this protein is reduced in rapidly proliferating cancer cells. Mass spectrometry data for GAS 2 (mean number of peptides 1 in SMNod and 7 in SMCtl) were not shown in Table 4-B. However, GAS 2 immunoblot data are shown in Figure 9.

**B.1.1.1.3 Equally expressed ER proteins in both fractions (control and tumor)**

- Valosine-containing protein (VCP/P 97) protein is equivalent to P97. This protein is involved in multiple signaling events including membrane fusion (Lavoie et al., 2000).

- Immunoglobulin heavy chain-binding protein/78 kDa glucose-regulated protein precursor (Bip/GRP 78) a chaperone protein was observed equally expressed between the control ER and tumor ER by immunoblot analysis. This confirms mass spectrometry results (described in details in Figure 6).

- Ribosomal protein S6 (ribo S6) is phosphorylated by a protein named ribosomal protein S6 kinase alpha-1 (Boylan et al., 2001). This protein is a key target of the Akt signaling pathway (Ruggero and Pandolfi, 2003).

In conclusion, these experiments were used for qualitative validation of mass spectrometry results. We were able to validate mass spectrometry results obtained from 14 different cancer related proteins.

### B.1.1.2 Validation of MS results using densitometry

Densitometric analysis was performed for both known and unknown proteins in four subcellular fractions, cytosol from control liver (S100Ctl), smooth microsomes from from control liver (SMCtl), smooth microsomes from dissected tumor nodules (SMNod), and cytosol from dissected tumor nodules (S100Nod).

### B.1.2 Endoplasmic reticulum and cytosol (control and tumor) fractions

Densitometry was carried out on immunoblots of fractions after using antibodies to p450 reductase, ATP citrate lyase, LRP and rAFAR 1 (Figure 10). We have decided to compare the relative amounts of proteins in both ER and cytosolic fractions. This was done because there are reports in the literature that confirm that cytosolic proteins can bind and interact with the ER (Rapoport, 2007). Therefore, we looked for change in the distribution of specific proteins in cytosol and ER fractions under control and tumor conditions.

**FIGURE 10: Proteins bands revealed by immunoblot.** Comparison of densitometric results of specific proteins in ER and cytosolic fractions from control rat liver and from dissected rat liver tumor nodules. 40 μg of fraction proteins were loaded on 7-15% (gradient) of SDS-PAGE. After protein separation, proteins were transferred onto nitrocellulose membranes (Bio-Rad). The protein blots were treated with primary antibody then with horseradish peroxidase enzyme tagged secondary antibody and revealed with enhanced chemiluminescence. The films were scanned and the density of the protein bands was determined using Scion Image densitometric software. Actual immunblots are shown from left to right for cytosol from control rat liver (S100Ctl), smooth microsomes from control rat liver (SMCtl), smooth microsomes from dissected liver tumor nodules (SMNod), and cytosol from dissected liver tumor nodules (S100Nod). Densitometric results are shown as bar values, which are aligned above the immunoblot results. Corresponding densities are expressed as arbitrary units, DA [1].

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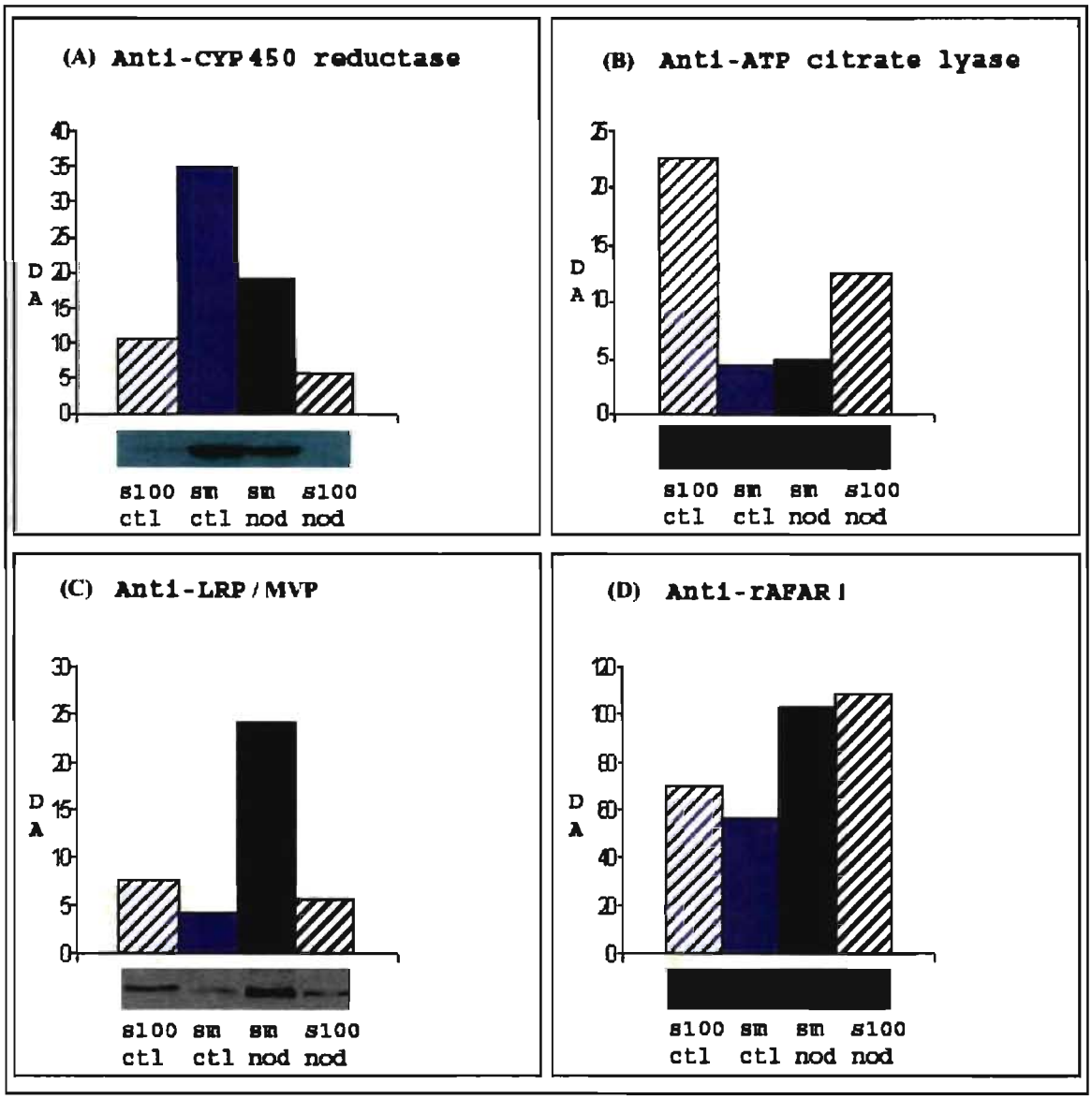
[1] Arbitrary density (DA). This was calculated after subtracting the background arbitrary density from each selected band, using Scion Image computer software program.


(A) Anti-CYP450 reductase
D A
40
35
30
25
20
15
10
5
0
s100 sm sm s100
ctl ctl nod nod
(B) Anti-ATP citrate lyase
D A
25
20
15
10
5
0
s100 sm sm s100
ctl ctl nod nod
(C) Anti-LRP/MVP
D A
30
25
20
15
10
5
0
s100 sm sm s100
ctl ctl nod nod
(D) Anti-rAFAR1
D A
120
100
80
60
40
20
0
s100 sm sm s100
ctl ctl nod nod

- Cytochrome p450 (CYP 450) reductase protein is strictly shown to be a membranous one (Figure 10-A). The CYP 450 reductase is a known integral membrane protein (Borgese and Pietrini, 1986). It is underexpressed in the ER tumor fraction. This confirms previous immunoblot data using only ER microsomes. The small amount in the cytosolic fraction probably corresponds to membrane contaminants. The contaminants could have arisen during cell fractionation or during proteins loading for electrophoresis.

- ATP citrate lyase is considered a cytosolic enzymatic protein (Elshourbagy et al., 1990). However, we have observed this protein in ER membranes by mass spectrometry (Table 4) and by immunoblot analysis (Figures 9 and 10-B). The amounts observed in the ER membranes were higher in tumor ER (Figures 9 and 10-B). Higher quantities of ATP citrate lyase were detected in the cyotsolic fractions (Figure 10-B) when amounts of this protein were compared in the two types of cytosolic fractions, the amounts in cytosol from tumor nodules was lower. This may be related to the physiological tumor state of the cells from which the cytosol originated. It is also tyrosine phosphorylated (Table 4).

- Lung resistance related protein/major vault protein (LRP/MVP) is considered a cytosolic protein (Sutovsky et al., 2005). However, we have detected it in ER fractions by mass spectrometry (Table 4) and by immunoblot analysis (Figure 10-C). We find LRP/MVP to be overexpressed in tumor ER (Figure 10-C). Here it is probably overexpressed in tumor ER after toxic treatment (aflatoxin). Lower amounts of LRP/MVP were observed in cytosolic fractions (Figure 10-C).

- Rat aflatoxin B1 aldehyde reductase 1 (rAFAR 1) protein is found in SM membranes and is overexpressed in tumour ER and overexpressed in tumor cytosol (Figure 10-D). This enzyme is well known to be overexpressed in liver after aflatoxin treatment (Ellis et al., 1993).

In conclusion, a variety of factors probably influences the amounts of specific proteins in cytosolic and ER fraction. These factors include whether the proteins are known to be integral membrane proteins (example CYP 450 reductase), whether the

proteins are known to be cytosolic (example ATP citrate lyase) or whether the proteins are known to be components of contaminating particles (example LRP/MVP).

### B.1.3 Comparison of densitometry of known proteins with mass spectrometry data for smooth membrane fractions

The amounts of specific proteins in smooth microsomes from control liver were compared quantitatively with the amounts of the same proteins in smooth microsomes from dissected tumor nodule using densitometry and using mass spectrometry. Figure 11 compares the ratios of the values obtained using the two techniques. For most proteins studied, the quantitative results obtained by densitometry were similar to the quantitative results obtained by mass spectrometry (Figure 11). Thus, the immunoblot data representing specific protein identification confirmed mass spectrometry data representing probability identification of the same proteins. This Western blotting validates the quantitative proteomics approach.

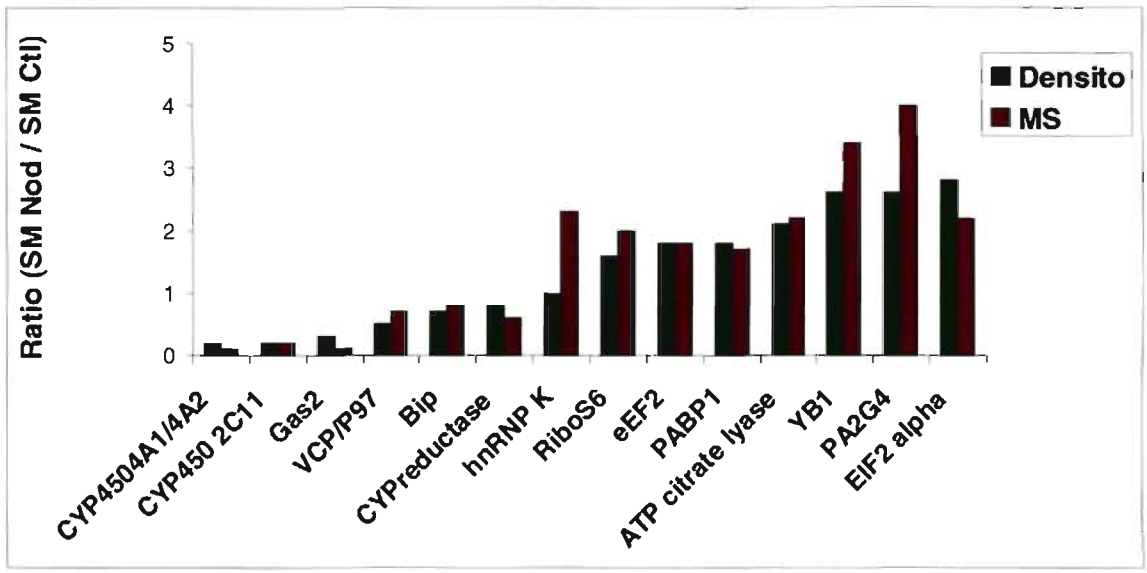



**FIGURE 11: Comparison of densitometry results with mass spectrometry results.** Amounts of specific proteins in using densitometry [1] and using mass spectrometry [2]. Amounts of proteins are expressed as ratios (amounts of protein in (SMNod) divided by amounts of protein in SMCtl) immunoblot data was obtained using antibodies against 14 specific proteins identified on the X-axis. Densitomteric analysis was carried out on the immunoblots [3] results shown in Figure 9. Densitometry data was used to determine ratios (crimson column). Mass spectrometry data was used to determine ratios of amounts for the same proteins (green column).

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[1] Densitomteric analysis was done using Image J computer software program.

[2] Quadriplet mass spectrometry experiments.

[3] Densitometry result for eEF 2 represented the mean of triplet measurements that come from two different experimental samples for SMCtl and SMNod membranes.

## B.2 Unknown proteins

### B.2.1 Studies with anti-ER antibodies

We have carried out immunoblot using anti-ER antibodies. The objective here was to compare amounts of proteins in membrane and cytosolic fractions from control rat liver and from dissected liver tumor nodules using anitserum that was raised against a purified ER fraction (Paiement and Roy, 1988). Using these antibodies in immunoblot and densitometry, we found that some proteins were underexpressed in tumor ER (Figure 11, band # 1) and other proteins were in higher concentration in tumor ER (Figure 11, band # 2). Some proteins were in similar concentrations in all fractions (Figure 11, band # 3) and some proteins were more concentrated in the ER fractions compared to those in the cytosolic fractions (Figure 11, band # 4).

**FIGURE 12: Densitometric analysis of proteins in fractions from control rat liver and from dissected liver tumor nodules using anti-ER antibodies. (A)** Primary polyclonal rabbit anti-ER was used to study unknown ER and cytosolic proteins in four experimental fractions, cytosolic proteins from control rat liver (S100Ctl), smooth microsomes from control rat liver (SMCtl), smooth microsomes from dissected liver tumor nodules (SMNod) and cytosolic proteins from dissected liver tumor nodules (S100Nod). Bands number 1 to 4 (arrows) were chosen for densitometric studies. Immunoblot was prepared as in Figure 10. **(B)** Actual immunblots are shown from left to right for cytosol from control rat liver (S100Ctl), smooth microsomes from control rat liver (SMCtl), smooth microsomes from dissected liver tumor nodules (SMNod), and cytosol from dissected liver tumor nodules (S100Nod). Densitometric results are shown as bar values, which are aligned above the immunoblot results. Corresponding densities are expressed as arbitrary units, DA [1].

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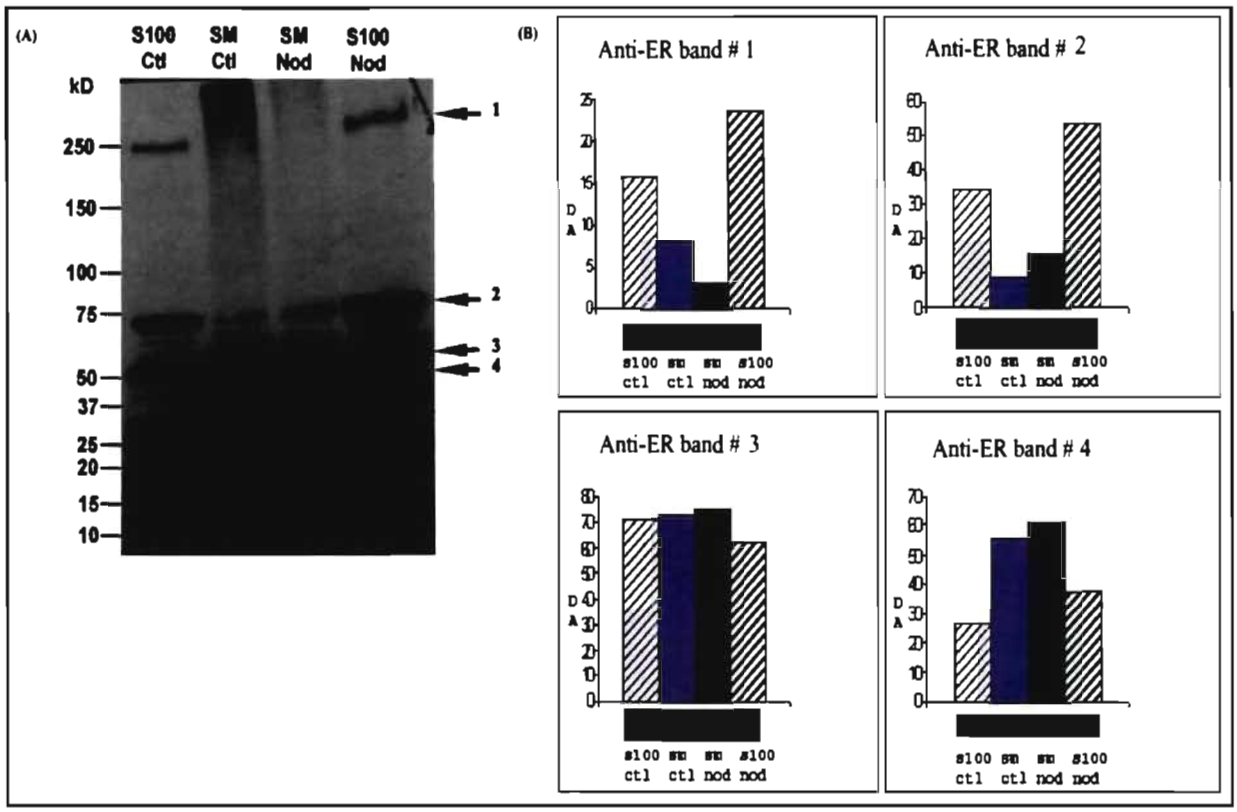
[1] Arbitrary density (DA). This was calculated after subtracting the background arbitrary density from each selected band using Scion Image computer software program.


(A)
S100 Ctl
SM Ctl
SM Nod
S100 Nod
kD
250
150
100
75
50
37
25
20
15
10
1
2
3
4
(B)
Anti-ER band # 1
Anti-ER band # 2
Anti-ER band # 3
Anti-ER band # 4
DA
s100 ctl
sm ctl
sm nod
s100 nod

In conclusion, as was observed in immunoblot studies using specific antibodies results obtained with anti-ER antibodies revealed differential expression of specific proteins (Figure 12, bands # 1 and 2) and some proteins did not change in concentration (Figure 12, bands 3 and 4). Since mass spectrometry identified many unknown proteins (Figure 8), the anti-ER antiserum could potentially be used to immunoprecipitate out such unknown proteins. This is because the anti-ER antibody recognizes a variety of proteins that have not been defined. The anti-ER antibodies could be used to immunoprecipitate ER proteins. Unknown proteins defined by MS could potentially be proteins that are recognized by the anti-ER antibodies. Finally, the cytosolic proteins that were detected using anti-ER antibodies are probably proteins that bind to specific ligands in the ER or they could be contaminating proteins of the ER fraction.

**B.2.2 Studies with anti-phosphotyrosine antibodies**

We have carried out immunoblot using anti-phosphotyrosine antibodies. The objective here was to compare amounts of proteins in membrane and cytosolic fractions from control rat liver and from dissected liver tumor nodules using anitserum that was raised against phosphsotyrosine residues (materials and methods, Table 1). Using these antibodies in immunoblot and densitometry, we found that some proteins were in higher concentration in tumor ER (Figure 13, bands # 4, 5 and 6) and that proteins were almost equally expressed in ER (Figure 13, band # 1, 2 and 3). Some proteins were more concentrated in the ER fractions compared to those in the cytosolic fractions (Figure 13, band # 4, 5 and 6).

**FIGURE 13: Densitometric analysis of proteins in fractions from control rat liver and from dissected liver tumor nodules using anti-phosphotyrosine (anti-PY) antibodies.** **(A)** Primary monoclonal mouse anti-phosphotyrosine (as identified in materials and methods, Table 1) was used to study unknown tyrosine phosphorylated ER and cytosolic proteins in four experimental fractions, cytosolic proteins from control rat liver (S100Ctl), smooth microsomes from control rat liver (SMCtl), smooth microsomes from dissected liver tumor nodules (SMNod) and cytosolic proteins from dissected liver tumor nodules (S100Nod). Bands number 1 to 6 (arrows) were chosen for densitometric studies. Immunoblot was prepared as in Figure 10. **(B)** Actual immunblots are shown from left to right for cytosol from control rat liver (S100Ctl), smooth microsomes from control rat liver (SMCtl), smooth microsomes from dissected liver tumor nodules (SMNod), and cytosol from dissected liver tumor nodules (S100Nod). Densitometric results are shown in graphic form. Densitometric results are shown as bar values, which are aligned above he immunoblot results. Corresponding densities are expressed as arbitrary units, DA [1].

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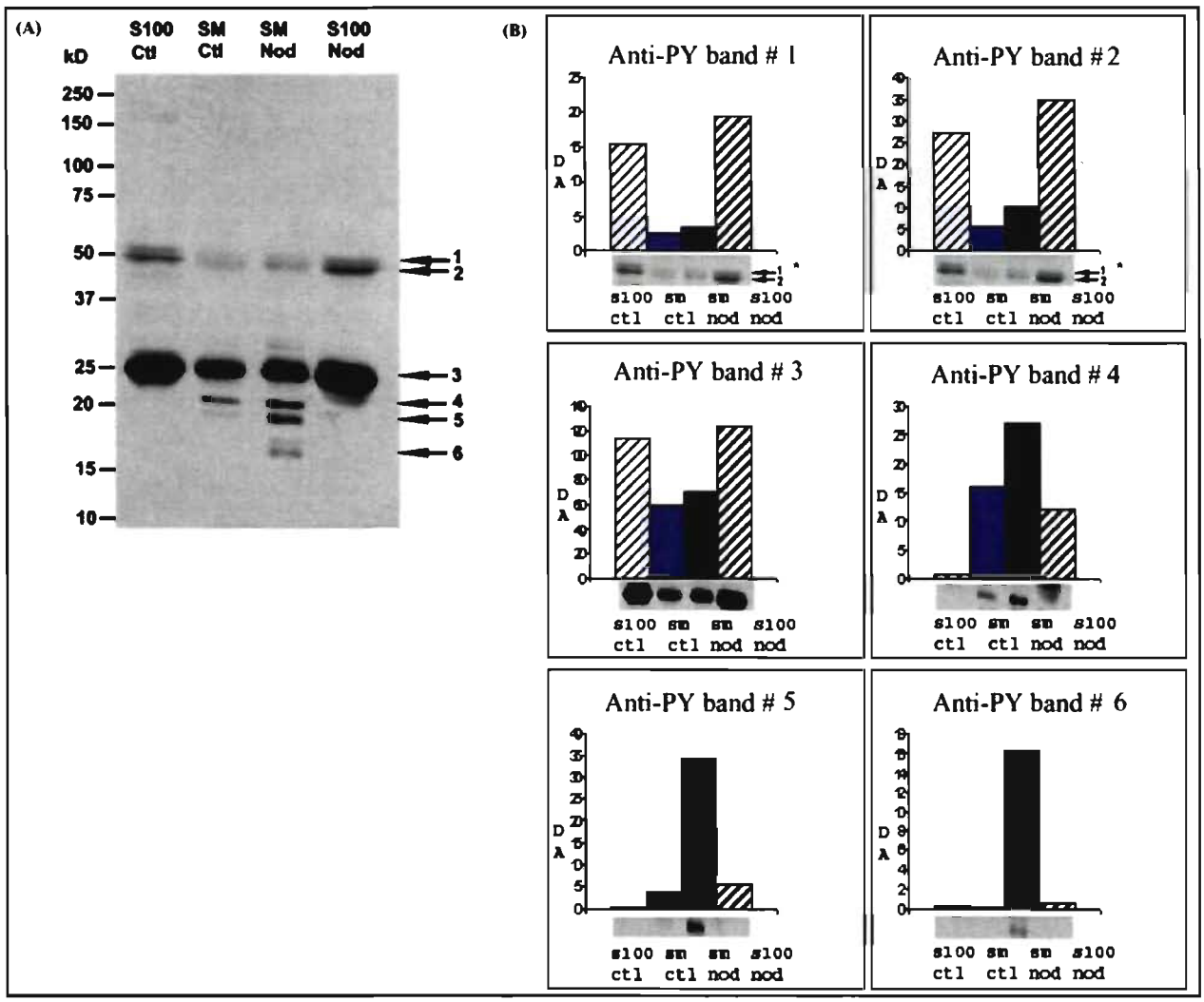
[1] Arbitrary density (DA). This was calculated after subtracting the background arbitrary density from each selected band using Scion Image computer software program.

* The bands numbers 1 and 2 were resolved by using a thin cursor width in the selection tool of Scion Image software program for densitometry.


(A)
S100 Ctl
SM Ctl
SM Nod
S100 Nod
kD
250
150
100
75
50
37
25
20
15
10
1
2
3
4
5
6
(B)
Anti-PY band # 1
Anti-PY band #2
Anti-PY band # 3
Anti-PY band # 4
Anti-PY band # 5
Anti-PY band # 6
D A
s100 sm sm s100
ctl ctl nod nod

In conclusion, as was observed in immunoblot studies using specific antibodies results obtained with anti-phosphotyrosine antibodies revealed differential expression of specific proteins (Figure 13, bands # 4, 5 and 6) and some proteins did not change in concentration (Figure 13, bands 1, 2 and 3). Since mass spectrometry identified many unknown proteins (Figure 8), the anti-phosphotyrosine antiserum could potentially be used to immunoprecipitate out such unknown proteins.

# DISCUSSION

The molecular machines of the ER in cancer are so complex and still vague. Proteomics analysis of ER and validation by other techniques such as the immunoblot, help partly in solving the mystery of the proteomic profile of ER in cancer (oncoproteome of ER). A key point to mention is that all cancer literature studied cells and tissues, while our study was done at the level of an organelle, the ER. Organelle proteomics is more sensitive and more precise than cell and tissue proteomics because protein complexity is largely reduced and is done to a range where an entire organelle-proteome can be displayed on a single 2D gel or even analyzed by mass spectrometry in gel-free procedures in one shot (Au et al., 2007; Brunet et al., 2003). In addition, identified proteins can immediately be linked to a functional context, because they were purified together with an organelle or subcellular fraction. Also, low abundant proteins and signaling complexes can be enriched. Analyzing subcellular fractions and organelles allows also tracking proteins that shuttle between different compartments, e.g. between the cytoplasm and nucleus (Au et al., 2007; Brunet et al., 2003). Importantly, subcellular fractionation is a flexible and adjustable approach that may be efficiently combined not only with two-dimensional gel electrophoresis but also with gel-independent techniques (Huber et al., 2003).

## A Differentially expressed proteins

### A.1 Proteins involved in mRNA metabolism

In our study, we were able to demonstrate many of proteins involved in mRNA metabolism as differentially expressed. Here we would like to highlight an important remark as regard one of them as follows:

- Heterogeneous nuclear ribonucleoprotein K (hnRNP K) as mentioned before belongs to hnRNPs family and is involved in mRNA metabolism (Habelhah et al., 2001). Since mRNAs are present in association with both free polysomes and membrane-bound

polysomes (Zambetti et al., 1987), therefore it is possible that hnRNP K is also attached to ER. In our study it was detected in the ER (Tables 2-A and 4-A as well as in Figure 9). It is also a phosphoprotein that can be phosphorylated on both tyrosine and serine residues (Feliers et al., 2007). It is an overexpressed protein in literature (Li et al., 2004). In addition, it has been reported as a potential new marker in colon cancer (Klimek-Tomczak et al., 2006).

**A.2 Proteins involved in diverse metabolic pathways**

In our study, we were able to demonstrate that many differentially expressed proteins are involved in diverse metabolic processes. Moreover, many of these proteins are either overexpressed or underexpressed in tumor ER. We would like to highlight some relevant points as follows:

- The pentose phosphate pathway (PPP) is involved in carbohydrate metabolism and it is composed of oxidative and non-oxidative branches. In the oxidative branch the hexose, glucose 6-phosphate is converted to pentose phosphate and carbon dioxide with the reduction of two molecules of nicotinamide adenine dinucleotide phosphate (NADP). While in the non-oxidative branch, three molecules of pentose phosphate (15 carbon atoms) are reconverted to two and one-half molecules of hexose phosphate (15 carbon atoms) in a series of fully reversible reactions (Horecker, 2002). Ribose-5-phosphate which is one of the major products of the PPP is important for the biosynthesis of nucleotides (Boren et al., 2006) and nucleic acids (Horecker, 2002). NADPH (NADP reduced form) is produced by the oxidative branch of the PPP and is used for detoxification processes and lipid biosynthesis (Boren et al., 2006). Enzymes involved in the non-oxidative branch of PPP include transaldolase, transketolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase. While enzymes involved in the oxidative branch of PPP include glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Boren et al., 2006; Wagner et al., 1978). As regard the non-oxidative branch of PPP, both transaldolase and the transketolase were overexpressed in our studies (see Tables 2-A and 4-A). Also, glucose 6-phosphate

dehydrogenase was uniquely expressed in tumor ER membrane in Table 4-A. Thus the PPP and glucose and glycogen metabolism are modified in cancer. These modified events in the context of the well-known Warburg effect in cancer cells include that cancer cells take up glucose at higher rates than normal tissue but use a smaller fraction of this glucose for oxidative phosphorylation. This effect is defined as aerobic glycolysis or the Warburg effect (Kim and Dang, 2006). This effect is thought to be caused by cancer leading to defects in oxidative phosphorylation, or "respiration" in the mitochondria, forcing the cancerous cell to revert to a more "primitive" form of energy generation (i.e. glycolysis). In his view, this switch caused such cells to become undifferentiated and cancerous (Garber, 2004).

- ATP citrate lyase is a substrate for protein kinase B (Akt) signaling pathway (Berwick et al., 2002). The Akt pathway has 7 distinct signaling pathways downstream and it promotes cell survival by inhibiting apoptosis. An important branch of the Akt pathway for cancer cell survival involves the kinase mTOR (mTOR is overexpressed in our mass spectrometry database with mean number of peptides is 2.5 in SMNod and 0.5 in SMCtl) (McCormick, 2004). Akt-transformed cells are dependent on ATP citrate lyase as a growth promoter through its role in de novo lipogenesis (Bauer et al., 2005). It is also tyrosine phosphorylated (Table 3) and previously shown in our data as overexpressed (Tables 4-A and 4, Figures 9 and 10-B).

**A.3 Proteins involved in aflatoxin B1 metabolism**

Aflatoxin B1 (AFB1) is a well-known hepatoxin and hepatocarcinogen (Bennett and Klich, 2003; Carnaghan, 1964). Following animal treatment with aflatoxin B1 (AFB1), many detoxification systems will be implicated to protect the organism against adverse effects of AFB1. Without such protection AFB1 treatment ultimately leads to development of hepatocellular carcinoma. However, the metabolism of AFB1 is complex. Drug metabolizing enzymes protect the body against harmful effects of different xenobiotics molecules (Xu et al., 2005). The enzymes involved in biotransformation of AFB1 belong to two main groups namely phase I and phase II

enzymes. Phase I (oxidation reactions) involves cytochrome p450 that catalyses monooxygenation reaction (Anzenbacher and Anzenbacherova, 2001) and aldo-keto-reductases 7A (aflatoxin aldehyde reductases) (Jin and Penning, 2007). While phase II (conjugation reactions) involves glutathione S transferase (Aliya et al., 2003). Many sub-family proteins that are implicated in AFB1 metabolism were detected in our data as differentially expressed. The important points of note are as follows:

1) Cytochrome p450 (CYP 450) family protein:

In eukaryotes, the members of this family are membranous proteins. Their basic function is in detoxification (Anzenbacher and Anzenbacherova, 2001). However, the resultant AFB 1 metabolites are sometimes more dangerous than the original molecule (Itoh et al., 1997). This can be more explained by the fact that cytochrome p450 is able to transform AFB 1 into aflatoxin-8,9-epoxide molecule (activated form) which is highly mutagenic and carcinogenic (Hayes et al., 1993; Loe et al., 1997). This activated form can interact with DNA leading to formation of DNA adduct (Smela et al., 2001). Thus, DNA mutations can affect p53, ultimately leading to liver cancers (Mace et al., 1997). However, this toxic effect can be avoided if the activated metabolite is reduced with phase 2 enzyme glutathione S transferase (Hayes et al., 1993).

As shown previously, many of p450 family proteins were underexpressed. These CYP 450 members include CYP 450 4A in Figures 9 and 11, CYP 450 2C11 in Table 4-B and Figure 9, CYP 450 reductase in Figures 9, 10-A and 11 and CYP 450 2C7, 3A18, 2A2, 2C6 and 2D2 in Table 4-B. In conclusion, many cytochrome p450 family members were underexpressed in tumor ER. This phenomenon may occur to prevent excessive formation of toxic metabolic intermediates.

2) Glutathione S transferase

Glutathione S transferase (GST) enzyme plays a protective role against AFB1-DNA adduct formation in tissues, i.e. causes detoxification of AFB1 (McLean and

Dutton, 1995). In our study we detected an important member of this family named glutathione S transferase, pi (GST pi) as one of the overexpressed proteins in tumor ER (Table 4-A). GST pi has been reported overexpressed in human HCC (Niu and Wang, 2005). It is has been proposed as a tumor marker for HCC in rat (Aliya et al., 2003) and for serodiagnosis in human HCC (Niitsu et al., 1989).

3) Aldo-keto reductases (aldehyde reductases) (AKR 7A)

Aldo-keto reductases (AKRs) are a cytosolic superfamily of proteins (Zeindl-Eberhart et al., 2001), which are NADPH oxidoreductases. These enzymes are phase I drug-metabolizing enzymes (Jin and Penning, 2007) that convert carbonyl groups to alcohols (Sawada et al., 1979). Hence, they can reduce the dialdehyde protein-binding form of aflatoxin B1 (AFB1) to the non-binding AFB1 dialcohol, this is contributed by the action of aflatoxin aldehyde reductases (AKR 7A) (Jin and Penning, 2007).

Among the members of this superfamily is the rat aflatoxin B1 aldehyde reductase (rAFAR 1) which was detected as an overexpressed protein as shown before in Table 4-A as well as in Figure 10-D. Rat aflatoxin B1 aldehyde reductase member 1 (rAFAR 1) is a well-known cytosolic protein and is involved in aflatoxin detoxification (Knight et al., 1999; Praml et al., 2003; Zeindl-Eberhart et al., 2001). Normally, in the rat two aldehyde reductase (AFAR) isoenzymes exist, called rAFAR 1 and rAFAR 2. The latter has been shown to be associated with Golgi membranes (Kelly et al., 2002). However, although rAFAR 1 is considered more a cytosolic protein, it may react with a specific ER substrate in cancer, indicating an affinity for ER membrane.

On the other hand, treatment with AFB1 will lead to overexpression of certain proteins, notably phase 2 enzymes (Yates et al., 2006), to protect the animal from serious adverse effects. Nrf 2 short form for NF-E2 related factor 2 is a member of a transcription factor family called nuclear transcription factor erythroid 2p45 (NF-E2) (Lee and Surh, 2005). Nrf 2 is involved in the regulation of aldo-keto reductase enzyme (Nishinaka and Yabe-Nishimura, 2005). In the cytoplasm, Nrf 2 is normally bound to Kelch-like ECH-

associated protein 1 (Keap 1) (Itoh et al., 1999; Itoh et al., 2003). Keap 1 represses Nrf 2 transcription activity by targeting it for ubiquitination and proteosome-mediated degradation (Zhang and Hannink, 2003). However, under certain conditions as the case of oxidative stress (Zhang and Hannink, 2003) Nrf 2 will able to escape from Keap 1 with subsequent nuclear translocation of Nrf 2 (Itoh et al., 1999; Itoh et al., 2003). In the nucleus Nrf2 is recruited to antioxidant response element (Nioi et al., 2003) and thus becomes able, to induce the expression of its target genes (Zhang and Hannink, 2003), which are, phase 2 enzyme genes (Itoh et al., 1997). Nrf 2 exerts a physiological role in cytoprotection due to toxic events (Kobayashi and Yamamoto, 2005). Downstream targets of Nrf 2 that were shown in our study to be overexpressed in tumor ER include GST pi (Table 4-A) (Ishii et al., 2000), glutamate-cysteine ligase (Table 2-A) (Yang et al., 2005), rAFAR 1 (Table 4-A and Figure 10-D) (Ellis et al., 2003), UDP-glucuronosyl transferase 1A6 (Table 4-A) (Bock and Kohle, 2005), microsomal epoxide hydrolase (Table 4-A) (Ramos-Gomez et al., 2001), glucose-6-phosphate dehydrogenase (Table 4-A), 6-phosphogluconate dehydrogenase (Table 4-A) (Thimmulappa et al., 2002).

## B Proteins that do not change in expression

### B.1 Folding proteins

The endoplasmic reticulum acts as a quality control system for newly synthesized proteins. Folding proteins are a group of specialised ER proteins whose main role is to monitor newly synthesised secretory or membrane proteins, so that correctly folded proteins are exported from the ER, but misfolded proteins are retained and selectively degraded (Hendershot, 2004). Figure 6 showed the most folding proteins were in equal concentration in control ER and tumor ER. This is found to be in contrast to previous studies that mentioned upregulation of members of folding proteins; Bip was previously reported as upregulated in human HCC (Luk et al., 2006) and in high grade human breast cancer (Fernandez et al., 2000). Why the presence of such remarkable disconcordance, is not known, but probable underlying mechanisms might be related to the immune escape mechanism (Luk et al., 2006) or the occurrence of metastases (Zhang et al., 2006), or the

development of drug resistance as the case in human breast cancer cells (Dong et al., 2005).

Another two important ER folding proteins we would like to discuss are the calnexin (an ER membrane protein) and calreticulin (an ER luminal protein) (Danilczyk et al., 2000). The calreticulin/calnexin cycle is important for the quality control function of ER (Wu et al., 2006). This is achieved by the binding of unfolded glycoprotein to calreticulin or calnexin. This result in retention of the unfolded glycoprotein in the ER, permitting foldases (include peptidylproline isomerases and disulfide isomerases) to catalyze the isomerization of polypeptide conformations or exchange of disulfide bonds to facilitate folding of the protein into the correct 3-D arrangement of protein structure (Schrag et al., 2003). The cancer literature reported the presence of anti-calreticulin autoantibodies in human HCC (Le Naour et al., 2002). On the other hand calnexin was reported by (Dissemond et al., 2004) as underexpressed in human metastatic melanoma (based upon an immunohistochemistry study). This study claimed a tumor immune surveillance escape mechanism to be responsible for their result. Moreover, their result is in concordance with our results (that detected underexpression of this protein in tumor ER, Figure 6).

**B.2 Proteins in the major histocompatibility class I (MHC class I) peptide loading complex (PLC)**

As regard the proteins which comprise the PLC, our results showed no significant change in expression between the tumor ER and the control ER. However, by reviewing the literature we found that our results are not consistent with other cancer studies. In cancer literature, many studies demonstrate underexpression of the PLC proteins. Examples include underexpression of β2-microglobulin due to genetic events in human lung and breast cancer (Chen et al., 1996). Other example is the underexpression of TAP 1 and TAP 2 in human melanoma; moreover, the underexpression of TAP 1 and TAP 2 markedly increased with progression of melanoma (Kageshita et al., 1999). This is due to suppressed function of related PLC proteins leading to underexpression of MHC class I

presentation of tumor-associated epitopes (Scholz and Tampe, 2005). This leads tumors to escape from T cell recognition with consequent escape cell death (Seliger et al., 2002). Nevertheless, underexpression of PLC components is not always evident in tumor cells since it is subjected to numerous intracellular factors (Park et al., 2006). Therefore, differential expression of PLC components may vary between tumor types or even during the stages of tumor development.

Thus, we can observe many differences that are present between our results and those reported in the literature. This can be partly attributed to two main factors, use of rat aflatoxin tumor nodules versus human tissues and metastatic versus non-metastatic samples. The rat tumors used in our studies were primary tumors at the time of sacrifice and tumor extraction with no evidence of metastasis was found (data not shown).

## C Phosphotyrosine proteins

In general, protein phosphorylation at tyrosine (Chang et al., 2008; Lewandrowski et al., 2008) or serine (Hacker and Karin, 2006) or threonine residues (Jaffe et al., 1998) plays an important regulatory role in diverse cellular functions. In our study we were able to observe several overexpressed proteins in tumor ER that were previously defined as tyrosine- phosphorylated (Rush et al., 2005). Such proteins might undergo cycles of tyrosine phosphorylation and dephosphorylation promoting specific ER related functions in cells, which are yet to be better defined (Lavoie and Paiement, 2008). On the other hand, phosphorylation cannot only be at tyrosine residues as shown in Table 4, but also at serine and/or threonine residues. Our data include examples of such overexpressed phosphoproteins: hnRNP K (Tables 2-A, 4 and Figure 9) that can be phosphorylated on both tyrosine and serine residues (Feliers et al., 2007), ATP citrate lyase (Tables 4-A and 3, Figures 9 and 10-B) (Ramakrishna et al., 1981) and elongation factor 2 (Tables 4-A and 3) (Kim et al., 1991; Ovchinnikov et al., 1990) that can be phosphorylated on tyrosine as well as serine and threonine residues.

On the other hand, phosphorylation is not just the simple addition of a phosphate group on specific protein residue (s) (Collins et al., 2007). In practise, this process

influences the activity of the phosphorylated protein itself (Manning et al., 2002). In our present study, we were able to detect overexpression of proteins related to diverse cellular functions (Table 3) and defined previously as being phosphorylated ones (Rush et al., 2005).

## D Protein contaminants of the ER fractions

The previously shown LRP/MVP in our study (Table 4 and Figure 10-C) is a major constituent of a ribonucleoproteins particle called vault (Poderycki et al., 2006). Vaults were originally discovered as contaminants of rat liver vesicle preparations (Kedersha and Rome, 1986). Therefore, we think that LRP/MVP is a constituent of vault particles that are purified coincidently with ER microsomes. Also, it is overexpressed in other types of cancer like colorectal one (Meijer et al., 1999) and under toxic conditions like drugs and toxic chemicals (Scheper et al., 1993).

Many proteins detected in the ER were cytosolic. Some of these cytosolic proteins are proteins that bind specifically to the ER (for example, ATP citrate lyase). However, some of the cytosolic proteins are likely ER contaminants produced during fractionation. This happens because there is a difficulty to obtain pure subcellular fractions (Huber et al., 2003). On the other hand, some cytosolic proteins could be associated with the ER because of yet determined protein-protein interaction?

## E Proteins with clinical relevance

In our studies, many of proteins were detected in our mass spectrometry bank database. By reviewing the literature, we found that some of them were clinically significant. Clinical significant proteins of note are as follows:

## E.1 Liver tumor biomarkers

In general, a tumour marker is a protein that can be detected in a solid tumor, in circulating tumour cells, in peripheral blood, in lymph nodes, in bone marrow, or in other body fluids (urine or stool). A tumour marker can be used for population screening and for detection, diagnosis, staging, prognosis, or follow up of cancer. Common laboratory methods for detection of a tumor marker include immunohistochemistry, fluorescent in situ hybridisation and reversed transcriptase and polymerase chain reaction (Lindblom and Liljegren, 2000). We now only talk about proteins that have been proposed as tumor markers by other scientists. Interestingly, we have observed many of these to be differentially expressed in our study.

Actually, there are now few available markers for liver cancer. Among the conventional serum biomarkers used in HCC diagnosis is the oncofetal protein namely alpha fetoprotein (AFP) which is the most widely used screening test in HCC (Zhou et al., 2006). However, this marker is non-specific as being detected in other types of cancers, AFP and testicular cancer (Arrieta et al., 2007; Lindblom and Liljegren, 2000). Moreover, AFP is not considered as a reliable biomarker for liver cancer since it is not increased in around 30% of HCC patients (Zhao et al., 2004; Zhou et al., 2006). So, the defective reliability of present liver tumor biomarkers highlights the importance to find out a new and more accurate HCC marker (s).

Our study revealed several overexpressed proteins considered in the literature to be potential biomarkers for HCC patients. These include eEF 2 (Figure 9) for early diagnosis (Li et al., 2008), nucleophosmin (Table 2-A) (Yun et al., 2007), GST pi (Table 4-A) (Aliya et al., 2003), transgelin 2 (Shi et al., 2005). For HCC metastasis, potential biomarkers include cytokeratin 19 (Table 4-A) (Ding et al., 2004; Marrero and Lok, 2004) and vimentin (Table 2-A) (Hu et al., 2004).

As regard transgelin 2, we found that it was overexpressed with mean number of peptides 2.75 in SMNod and 0.25 in SMCtl.

## E.2 Proteins related to tumor therapy

### E.2.1 Dihydropyrimidine dehydrogenase deficiency

Dihydropyrimidine dehydrogenase (DPD) is an enzymatic protein that was detected in our mass spectrometry studies and found to be an underexpressed protein in tumor ER (mean number of peptides were 1 in SMNod and 4 in SMCtl). This protein is a rate-limiting enzyme in the catabolism of the pyrimidine bases; it catalyses the reduction of thymine and uracil to 5,6-dihydrothymine and 5,6-dihydrouracil, respectively (Van Kuilenburg et al., 1998). A number of cancers are treated with 5-fluorouracil (Van Kuilenburg, 2006). This leads to inhibition of nucleotide metabolism and consequently affects cell division of tumor cells (Schuetz et al., 1986). Under normal condition DPD metabolizes 5-fluorracil (5-FU), but in patients that are deficient in DPD toxic amounts of 5-FU accumulate and can kill patients. Since DPD has been reported underexpressed in cancer (confirmed in our mass spectrometry studies) treatment of all cancers with, 5-FU should always be done with prior screening of DPD deficiency.

### E.2.2 Proteins involved in multiple drug resistance

Multiple drug resistance (MDR) state reflects the presence of resistance to treatment with multiple chemotherapeutic agents in cancer patients. Several well known transmembrane proteins are involved in drug resistance (Tan et al., 2000). Lung resistance related protein/major vault protein (LRP/MVP) is an example of a MDR protein (Shi et al., 2008; Tan et al., 2000) that was initially discovered in a non-small-cell lung cancer (NSLC) (Scheper et al., 1993). In our results, it was observed as an overexpressed protein (Figure 10-C) and it has been shown to be tyrosine-phosphorylated (Table 4). Upregulation of MVP is a predictor of the MDR phenotype (Herrmann et al., 1999) and hence a prediction of resistance to anticancer drugs (Ikeda et al., 2008).

### E.2.3 The Warburg effect and cancer therapy

The cancer phenotype that was described initially by Warburg as an increased cellular glycolysis in a cancer environment provides an important biochemical basis for the design of multiple anticancer therapeutic agents. The glycolytic pathway is a series of metabolic reactions catalyzed by multiple enzymes, the latter represent possible targets for the development of glycolytic inhibitors. Many of these inhibitors are currently at various stages of pre-clinical and clinical studies. These inhibitors may be particularly useful for the treatment of cancer (Chen et al., 2007; Giaccone et al., 2004).

# CONCLUSIONS AND FUTURE PERSPECTIVES

## Conclusions

Cancer may be characterized as the occurrence of complex molecular events at the cellular level. This disease results in interference with multiple cellular functions and can lead to the inhibition of apoptosis, which is the immortalization of cells.

The protein composition of the ER of hepatocellular carcinoma is different from that of the ER of a normal liver. These differences are summarized in the following three main points, which are:

I) a molecular signature of the ER in hepatocellular carcinoma.

- Metabolic profile alterations in cancer as evidenced by differential expression of many metabolic proteins, these include:
    1) Overexpression of proteins involved in glucose metabolism (PPP), which include transaldolase and transketolase;
    2) Underexpression of proteins involved in glycogen metabolism; and
    3) Underexpression of proteins involved in lipid metabolism.

- Differential expression of proteins involved in detoxification of AFB1 as evidenced by differential expression of many proteins belonging to different protein families involved in detoxification. These include:
    1) Underexpression members of cytochrome p450 protein family; and
    2) Overexpression of glutathione-S-transferase and aldo-keto reducatses.

- Overexpression of anti-apoptotic proteins. These proteins may lead to loss or alteration in the programmed cell death in cancer. This will favor cancer growth and metastases.

- Overexpression of proteins involved in mRNA metabolism. These proteins may lead to altered effect on the expression of different normally functional genes in tumor.

- Overexpression of proteins involved in ribosomes biogenesis.

- Overexpression of tyrosine phosphoproteins. These proteins may be implicated in signaling of diverse metabolic pathways.

II) Differential regulation of anti-apoptotic proteins (examples include tripeptidyl peptidase II and nucleophosmin) may in part explain why microinjection of ER membranes from adult rat liver into blastomeres of Xenopus embryos led to inhibition of cellular division in the blastomeres, whereas microinjection of ER membranes from liver nodules of rats treated with aflatoxin B1 did not (Paiement et al., 1992). However, in order to confirm this, injection of anti-apoptotic elements in blastomeres should be done.

III) The study of ER proteomics has led us to discover a set of new molecular screening markers for HCC, many of which need clinical validation.

Finally, this proteomic difference between control and tumor liver may be beneficial to the survival of the tumoral cells in liver. Moreover, the importance of these results will help us to understand better the role of the ER in cases of hepatocellular carcinoma.

Our results therefore suggest that many proteins reported in scientific literature detect very fewer ER proteins in tumor cells and tissues. This highlights the importance of our study and the contribution it could have in the field of cancer proteomics.

## Future perspectives and challenges

From our study, an important argument can be derived. In reviewing pre-existing studies, it was found that the concept of immune surveillance escape mechanism is due to

the differential expression of proteins involved in folding and in PLC in HCC. However, our study argues that this concept is not well supported, as we found no evidence of significant differential expression in the said proteins.

The two most important promising prospective is to identify more and new serum markers for the better detection and diagnosis of HCC, as well as to better identify the process of phosphorylation of proteins in HCC. Our study found that the ER proteomic expression profile in HCC could help in the future selection of proteins as candidate markers for the diagnosis of HCC. Moreover, our list of the newly discovered ER proteins, which we have designated as NERA, represents a panel for detecting potential new liver tumor biomarkers in the future. However, further study is needed to characterise these proteins.

On the other hand, the role of phosphorylation in binding cytosolic proteins to ER and the proper ER functions in HCC also has to be studied and investigated in the future. This can be achieved by using ER phosphoproteomics. However, phosphoproteomics has the advantage of reducing the amount of proteins that can be analyzed even though there is difficulty in the identification of phosphorylated proteins (Zhang et al., 2007). Moreover, the role of phosphorylation of proteins and ER membrane association should be considered in future studies.

The final perspective of this work is that the comparative study of the protein expression in HCC and the normal liver tissue may help us to find tumor-specific proteins that can be responsible for uncovering altered metabolic pathways. This study can help us to better understand the liver cancer process in rodents.

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***"Our heart is a cave in which secrets and wonders are kept, one of them is the secret of our reality and truth. In the cell, the endoplasmic reticulum does the same in being the heart of the cell".***