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Research Article

A Different Trend of Heat Shock Proteins 90 mRNA and Protein Inhepatocellular Carcinoma Cell Line-Secreted Extracellular Vesicles

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Abstract

Primary Hepatocellular Carcinoma (HCC) does not usually show any symptoms at the early stage and the use of biomarkers is necessary to aid in diagnosis. Recently Extracellular Vesicles (EVs), submicron membrane-bound structures secreted from different cell types containing a wide variety of bioactive molecules, have increased the attention in many cancers, including HCC, becoming an auspicious candidate as biomarkers and therapy in the scenario of limited diagnostic and treatment option.

Many indications have shown that heat shock proteins (Hsps) are important modulators in treatment resistance and invasion of HCC becoming attractive therapeutic targets. In particular, Hsp90α/β isoforms have been found to play critical roles in regulating the proliferation, apoptosis, and metastasis of tumor cells, suggesting for these proteins a role as targets for modern anticancer therapies. The study aimedto verify the presence of Hsp90α/β in EVs secreted by an HCC tumor cell line (HepG2) and by a non-tumorigenic hepatocyte cell line (WRL68), both at protein and mRNA levels, and to analyze their expression variations. The result showed that Hsp90s are transported by the EVs as protein but not at the mRNA level. To build new therapeutic targets using EVs or other organelles as performed on exosomes in recent studies, it is essential to evaluate the action at the pre or post-transcriptional level given their different behavior in transporting proteins or mRNA.

Keywords: HCC; Extracellular vesicles; Hsps; Real-time PCR; Protein analysis

Introduction

Primary Hepatocellular Carcinoma (HCC), one of the most common malignant tumors worldwide, does not usually show any symptoms at the early stage. By the time clinical manifestations appear, most patients have entered the terminal stage with fast and aggressive tumor progression; therefore, HCC screening and diagnosis are of extreme importance and the use of biomarkers is necessary to aid in diagnosis.

Recently Extracellular Vesicles (EVs), submicron membrane-bound structures secreted from different cell types containing a wide variety of bioactive molecules, have increased the attention in many cancers, including HCC, becoming an auspicious candidate as biomarkers and therapy in the scenario of limited diagnostic and treatment options [1,2]. EVs are commonly used by normal and tumor cells for communication at long distances to exchange complex molecular messages and deliver a variety of essential biomolecules [3]. The contents of vesicles vary concerning the mode of biogenesis, cell type, and physiologic conditions. In general, all EVs are loaded with various proteins, lipids, and nucleic acids [4] able to reprogram target cells to promote tumor growth, migration, metastasis, immune evasion, or chemotherapy resistance. Moreover, engineered EVs may be utilized as therapeutic agents, improving treatment options [5]. In recent years, many indications have shown that heat shock proteins (Hsps) are important modulators in treatment resistance and invasion of HCC, and novel therapeutic strategies that target Hsps alone or combined with other anticancer agents are widely investigated [6,7] also using EVs [8]. The Hsps are a group of highly conserved molecular chaperones acting in cell function including protein folding, assembly of the protein complex, and protein degradation [9].They are expressed at low levels under normal conditions while they increased in response to cellular stresses, including heat shock, hypoxia, genotoxic agents, nutrient starvation, and over expression of oncoproteins [10-13]. In particular, Hsp90, a member of the Hsp family, has been found to play a critical role in regulating the proliferation, apoptosis, and metastasis of tumor cells [14,15]. The Hsp90 family has four major members: Hsp90α, Hsp90β, GRP94, and Hsp75 [16,17]. Hsp90α and Hsp90β are located mainly in the cytoplasm, while the other two proteins in the endoplasmic reticulum and mitochondrial matrix, respectively. Due to its key role in modulating signal transduction, especially in tumor cells, Hsp90α has become a research hotspot. A recent study showed that plasma Hsp90α can discriminate patients with liver cancer from non-liver cancer controls [18]. Some reports showed that Hsp90α could be actively translocated into the extracellular space by malignant tumor cells [19]. In addition, the Hsp90α plasma level of patients with malignant tumors increased significantly and correlated positively with the degree of malignancy and the ability of producing metastasis

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[20]. Although most studies account for the effects of the Hsp90α isoform on angiogenesis, the role and mechanism of the Hsp90β isoform in tumor angiogenesis are rarely mentioned. Hsp90β is associated with the tumor malignancy of hepatocellular carcinomas and was up-regulated in HCCs with a high degree of malignancy [16]. Apart from their cytoprotective/antiapoptotic roles in the cytosol, Hsps have been found to provide danger signals for the host's cellular immune system when located in the extracellular space or on the plasmamembrane [21-25]. These findings suggest that Hsps may be an ideal candidate for enhancing antitumor immunity and there is an increasing interest in identifying the extracellular activities of different Hsps, prompting a consistent effort to study these proteins as targets for modern anticancer therapies. Indeed, understanding these events would be particularly relevant for designing EV-based therapeutic approaches. Nevertheless, due to the complexity of the pathways involved, the internalization route and fate of EVs inside recipient cells remain to be fully elucidated. The use of EVs in cancer therapy represents one of the future challenges for emerging therapeutic applications of EVs. Although it has long been known that EVs carry numerous Hsps that have a bioactive effect on target cells, as mentioned above, and some studies and clinical trials have focused on inhibitors of these proteins as anti-cancer therapies [26–29], few data are available or ongoing on the Hsps carried by EVs. Given the important role of Hsp90α and Hsp90β in tumor progression and cancer cell proliferation, including HCC, they could be good candidates for this purpose but first of all, it is useful to verify their presence in the EVs both at the protein level and mRNA.

For this reason, we aimed to verify, by protein and transcriptional study, the presence of Hsp90α and Hsp90β in EVs secreted by an HCC tumor cell line (HepG2), and by a non-tumorigenic hepatocyte cell line (WRL68), and to analyze their expression variations in both these EVs.

Materials and Methods

Cell Culture and Isolation of EVs by Differential Centrifugation

This study is a part of a larger project within which the transcriptional profile of potential regulating miRNAs/lncRNAs and novel molecular diagnostic markers of HCC were also evaluated in the same samples [30]. As previously reported [30], the human HepG2 HCC cell line (Sigma-Aldrich, St. Louis, MO, USA) and the human WRL68 normal hepatocyte cell line (Sigma-Aldrich) were cultured a dedicated enriched medium (Sigma-Aldrich Life Technologies).

After adding containing EV-depleted FCS (Life Technologies), EVs have been isolated by the supernatant of each cell line, through differential centrifugation [31]. Analysis of optical microscopy images does not support the presence of HeLa cells in our cell samples.

Protein Extraction and MS Analysis

EV proteins were extracted as previously reported [32]. Protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) and 100 µg of proteins were treated for high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis, as previously described [32].

We used an Information Dependent Acquisition (IDA) tandem mass spectrometry approach based on a survey MS1 scan followed by the selection of a maximum of 20 most abundant precursor ions and their further fragmentation by Collisional Induced Dissociation (CID) to generate MS2 spectra.

Raw peptide MS data were converted into a peak list format (mzML, centroid spectra) using the Proteo Wizard tool ms convert and searched against a reviewed human database (UniProtKB/Swiss-Prot, 20381 sequences, release February 2021) using the integration of X!Tandem and Comet search tools through the Trans-Proteomic Pipeline (TPP) software suite [33]. MS1 full-scan filtering workflow of Skyline software (version 21.1, McCoss Lab, University of Washington, USA) was used to extract and integrate the area under the peak curve of all Hsp detected peptides. Peptides abundances were integrated to obtain the protein abundances of Hsp90α (P07900) and Hsp90β (P08238) within Skyline software.

Transcriptional Analysis

Transcriptional analysis of Hsp90α and Hsp90β was carried out in the in vitro model WRL68 normal hepatocyte (n=6) vs. HepG2 HCC cell line (n=6), then in EVs isolated by both of them. As previously reported [30], the purification of RNA from both cell lines and EVs isolated by HepG2and WRL68 cell culture was carried out using acid guanidinium thiocyanate–phenol-chloroform method (Qiazol, Qiagen SpA, Milano, Italy) following miRN easy Mini kit manufacturer's instruction (Qiagen SpA, Milano, Italy). High-quality RNA was then eluted in 15-30 µl of RNAse-free water [30]. The total RNA concentration was determined in all samples by measuring the spectrophotometer absorbance (Nano drop, ThermoFisher). The RNA samples were stored at -80°C for use in gene expression studies. Total RNA extracted from all samples (cells and EVs) was reverse transcribed with miScript II RT Kit (Qiagen SpA, Milano, Italy). The cDNA samples obtained were stored at 4°C until Real-Time PCR analysis that was performed in duplicate in the Bio-Rad C1000™ thermal cycler (CFX-96 Real-Time PCR detection systems, Bio-Rad Laboratories Inc., Hercules, CA, USA) [24,25] using a specific fluorogenic DNA binding dye. The optimal Real-Time PCR conditions and the linear standard curves were developed for each gene analyzed. In order to verify the specificity of the amplification products, the amplicons were tested through melting curves analysis.

Intron-spanning primers were selected to avoid amplification of genomic DNA. The primers for reference (PPIA, TPT1, RPS4X eEF1a, RPL13a) and the target genes (Hsp90α, Hsp90β), were designed with a specific software Beacon Designer® (version 8.1;Premier Biosoft International, PaloAlto, CA) (Table 1) and were synthesized by Sigma Aldrich (Merck KGaA, Darmstadt, Germany) (Table 1).

Statistical Analysis

This study was carried out to conform to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments [34]. The reference genes were run in each system group analyzed: 1) EVs isolated byWRL68 and HepG2 HCC cell line2) WRL68 and HepG2 HCC cell line cultures 3) WRL68, HepG2 HCC cell line and EV secreted by them. For each system we found as reference genes: 1) PPIA, TPT1, RPS4X 2) eEF1a, RPL13a, RPS4X3) PPIA, TPT1, RPS4Xrespectively (M<1).

Relative quantification of each target gene studied was calculated by the ΔΔCt method. Group comparison was performed by Student's

Table 1: Primer sequence details of the analyzed gene.

Genes	*Primer sequence*	*GenBank, accession n.*	*Length (pb)*	*Temp (°C)*	*Efficiency (%)*	*R^2*
eEF1a	F:CTTTGGGTCGCTTTGCTGTT R: CCGTTCTTCCACCACTGATT	NM_001402	183	60	101.7	0.998
RPL13a	F:CGCCCTACGACAAGAAAAAG R: CCGTAGCCTCATGAGCTGTT	NM_012423	206	60	104	0.999
RPS4X	F:GATCCCCTCATCAAGGTGAA R: GCCCTTGCCAATAACAAAAA	NM_002046	243	60	104.2	0.999
PPIA	F:CTTGGGCCGCGTCTCCTTCG R: TTGGGAACCGTTTGTGTTTGGGGC	NM_021130	285	60	103.4	0.998
TPT1	F: AAATGTTAACAAATGTGGCAATT R: AACAATGCCTCCACTCCAAA	NM_003295	164	60	105	0.999
Hsp90a	F: CCTACTGCTGATGATACCA R: AGCCAGAGATTAGTCTACTTC	NM_001040060	102	60	95.5	0.995
Hsp90b	F: CTCTCCTGTCCTTGTGTTG R: CATCCAATCCTGCTGTCAA	NM_001271969	82	60	97.3	0.996

eEF1a: Eukaryotic translationelongation factor 1 alpha 1; **RPL13a:** Ribosomal protein L13a; **RPS4X:** 40S ribosomal protein S4, X isoform; **PPIA:** peptidylpropyl isomerase A [cyclophilin A]; **TPT1:** tumor protein translationally controlled 1; **Hsp90α:** heat shock protein 90 alpha; **Hsp90β:** heat shock protein 90 beta.

t-test or analysis of variance (ANOVA) as appropriate, using Statview 5.0.1 software released for Windows Statistical (SAS Institute, Inc., Cary, NC, USA). Relations between variables were assessed by linear regression. The results were expressed as mean ± SEM, and the *p-value* was considered significant when < 0.05.

Results

Protein Expression Analysis

Hsp90α (P07900) and Hsp90β (P08238) proteins were identified and quantified in WRL68- and HepG2-derived EVs with an average variability coefficient of 6.3% and 6.8% respectively. In both cases, proteins resulted increase in EVs secreted by the HCC tumor cell line (Figure 1), with a mean ratio of 2.7 for Hsp90α and 7.5 for Hsp90 β with respect to EVs secreted by non-tumorigenic hepatocyte cell line WRL68.

Gene Expression Analysis

We evaluated the Hsp90α and Hsp90β mRNA levels in EVs secreted by HepG2, the tumorigenic hepatocyte cell line, and by the non-tumorigenic hepatocyte cell line WRL68, and unexpectedly their transcript resulted to be almost undetectable in EVs secreted by the HCC tumor cell line (Figure 2).

A significant correlation was observed between Hsp90α and Hsp90β mRNA (r=0.990, p<0.001).

To have a complete as possible picture of the Hsp90α and Hsp90β mRNA expression trend in the tumorigenesis we also evaluated their mRNA expression levels in HepG2 and WLR68 cell lines. Both of them resulted to be higher in the tumorigenic cell line HepG2 with respect to the normal cell line WLR68 reaching significant levels only for Hsp90β (Figure 3).

Sharing the transcriptional data as a whole (WRL68, HepG2 HCC cell line, and EV secreted by them) we can observe that both the Hsp90α (Figure 4a) and the Hsp90β mRNA (Figure 4b) resulted being present in both cell lines and EVs-derived WRL68, while were undetectable in HepG2-derived EVs, as if the EVs secreted by HepG2 carry neither the Hsp90α mRNA nor the Hsp90β mRNA.

Discussion

Heat shock proteins are evolutionally conserved and ubiquitously expressed molecular chaperones abundantly present in cancer [35-37]. Recent gene expression studies have shown that Hsps can be used as prognostic markers to predict the clinical outcome of breast cancer patients [38]. Furthermore, the discovery of the extracellular vesiclesrole in transferring protein and genetic information and the identification of Hsps in EVs have opened new opportunities and

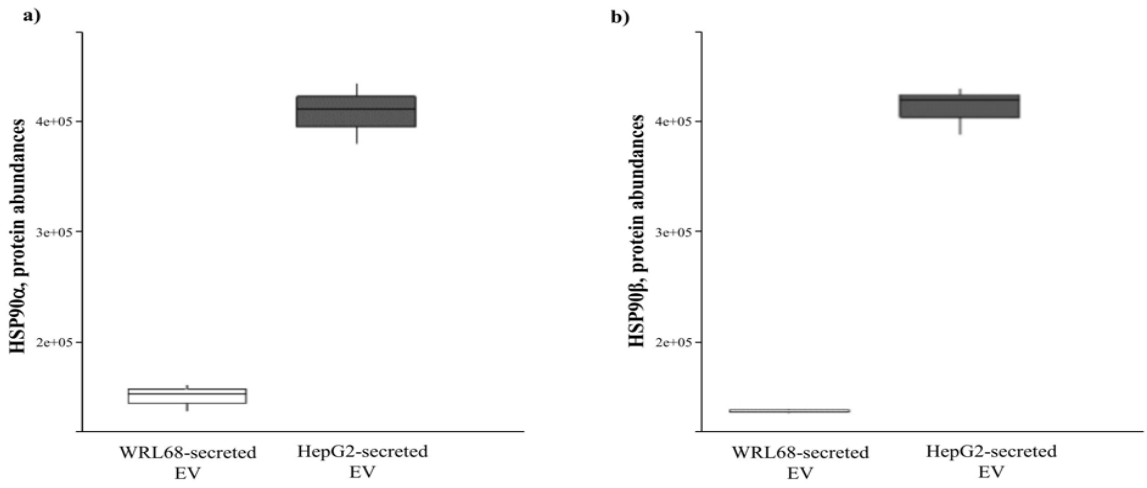

Figure 1: a) Hsp90α and b) Hsp90β protein levels in EVs secreted by non-tumorigenic hepatocyte cell line WRL68 (white bar) and by tumorigenic hepatocyte cell line HepG2 (grey bar).

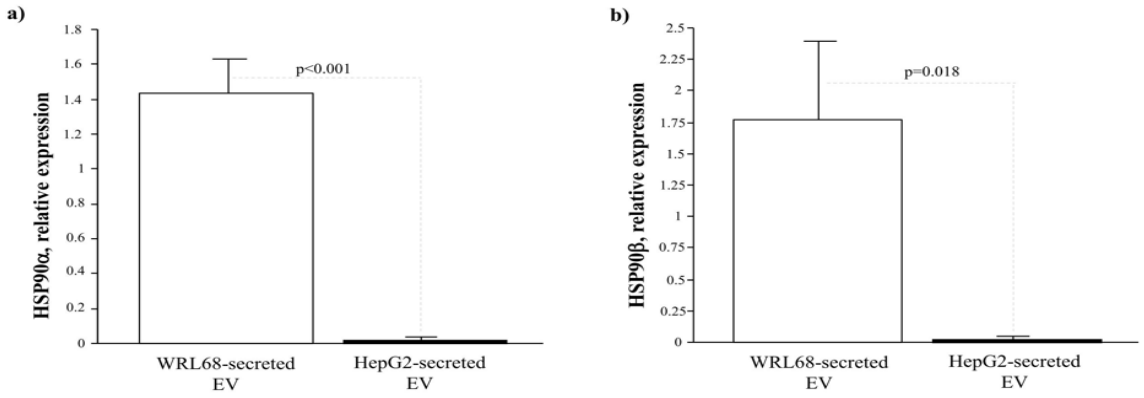

Figure 2: a) Hsp90α and b) Hsp90βmRNA levels in EVs secreted by non-tumorigenic hepatocyte cell line WRL68 (white bar) and by tumorigenic hepatocyte cell line HepG2 (grey bar).

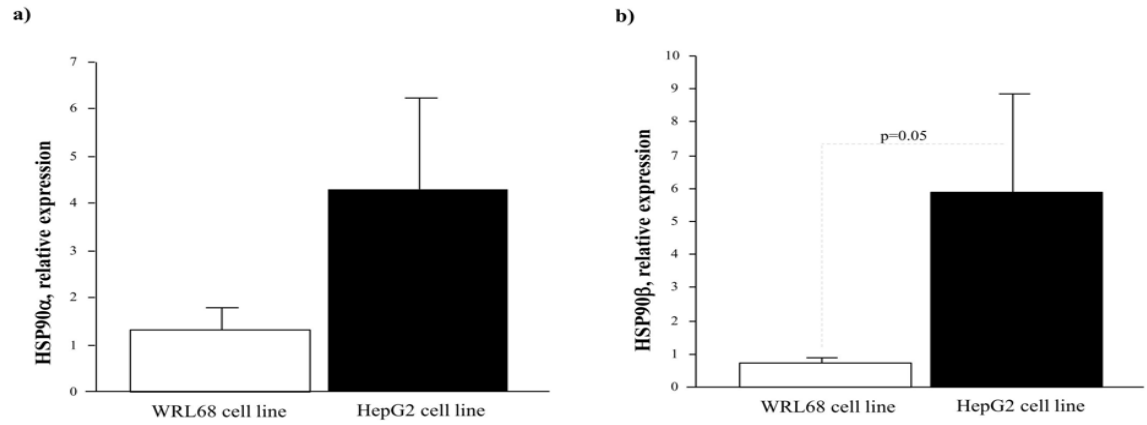

Figure 3: a) Hsp90α and b) Hsp90βmRNA levels in non-tumorigenic hepatocyte cell line WRL68 (white bar) and in tumorigenic hepatocyte cell line HepG2 (grey bar).

challenges for determining clinical biomarkers of cancer [25].

EVs, as physiological mediators of intercellular communication [23-25], are natural modulators of the gene expression of their target cells, and this feature could be a useful tool for diagnostic and therapeutic approaches. As also above mentioned Hsps may serve as diagnostic and prognostic markers in HCC [6,7] but there are still some challenges to target Hsps in HCC and to understand if their identification in EVs can open new opportunities and trials for determining clinical biomarkers of cancer or therapeutic target.

The present study provides a twofold indication: on the one hand, the increase in expression mRNA levels of Hsp90α and Hsp90β in the tumorigenic cell line is pointed out and in line with the literature data, on the other hand, an original result was obtained about the study of Hsp90α and Hsp90β in EVs secreted by tumorigenic and non-tumorigenic cell lines which highlights an increase in protein concentrations but not of mRNA expression. As previously reported,this study is a part of a larger project within which the transcriptional profile of potential regulating miRNAs/lnc RNAs [30] and novel molecular diagnostic markers of HCC were also evaluated in the same samples and, as observed in a previous study of ours [30], not all the biomarkers have, in the EVs secreted by tumorigenic cells, an up-regulation with respect to EVs extracted from non-tumorigenic cellsunderlining that for some biomarkers, the transcriptional and protein results may not have the same trend underlining different behaviors for EVs obtained from tumorigenic cells. In particular, the results obtained in this study indicated that the Hsp90s are transported by the EVs at the protein level but not at the mRNA level, highlighting the need to make specific choices also in their use as a therapeutic target. It has been suggested that EV cargos cannot also be completely reflective of their cell origin, and the underlying mechanism of cargo sorting is complicated and need to be further elucidated [39].

Conclusions

In order to build new therapeutic targets using EVs or other organelles as performed on exosomes in recent studies [8], it is essential to evaluate the action at the pre or post-transcriptional level given their different behavior in transporting proteins or mRNA.

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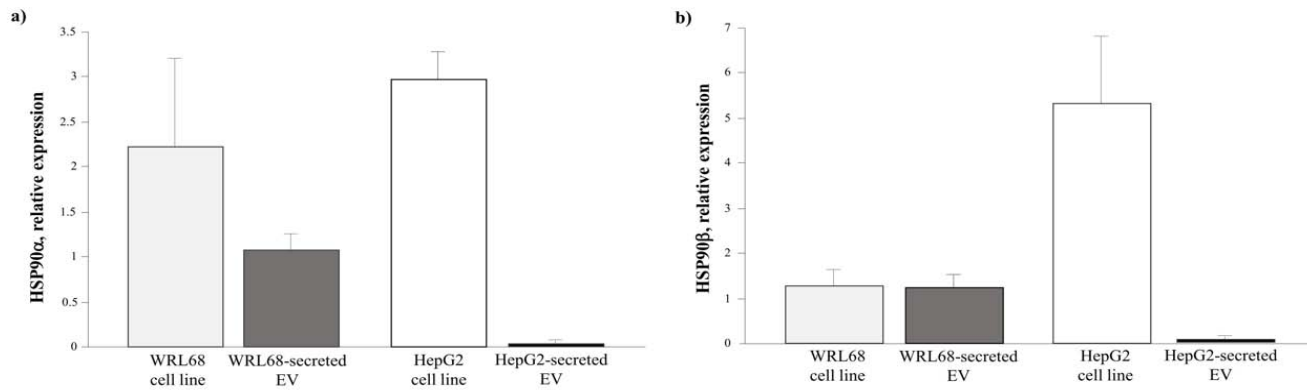

Figure 4: a) Hsp90α and b) Hsp90βmRNA levels in WRL68 and in HepG2 cell line compared with their levels in EVs secreted by them, respectively.

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Conflicts of Interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Consent for Publication

All authors gave their consent for this paper publication.

Author Contributions

All authors contributed to the study conception and design. Conceptualization, data curation, formal analysis: SDR, SR. Methodology: MC, NdG, SdT, CC, AC. Writing - original draft: SDR. All authors reviewed the manuscript.

All authors read and approved the final manuscript.

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