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Expression profile of adrenomedullin and its specific receptors in liver tissues from patients with hepatocellular carcinoma and in tumorigenic cell line-secreted extracellular vesicles

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ABSTRACT

The transcriptional profile of adrenomedullin (AM), a new metastasis-related factor involved in hepatocellular carcinoma (HCC), and its specific receptors (CLR, RAMP1, RAMP3) were evaluated in liver tissues of HCV-positive HCC subjects undergoing liver transplantation (LR) and in donors (LD). AM and its specific receptor expression were also assessed in extracellular vesicles (EVs) secreted by tumorigenic (HepG2) and non-tumorigenic (WRL68) cells by Real-Time PCR. AM expression resulted significantly elevated in LR concerning LD ($p = 0.0038$) and, for the first time, significantly higher levels in HCC patients as a function of clinical severity (MELD score), were observed. RAMP3 and CLR expression increased in LR as a function of clinical severity while RAMP1 decreased. Positive correlations were found among AM, its receptors, and apoptotic markers. No AM mRNA expression difference was observed between HepG2 and WRL68 EVs. RAMP1 and RAMP3 resulted lower in HepG2 concerning WRL68 while significantly higher levels were observed for CLR. While results at tissue level characterize AM as a regulator of carcinogenesis-tumor progression, those obtained in EVs do not indicate AM as a target candidate, neither as a pathological biomarker nor as a marker involved in cancer therapy.

1. Introduction

At present, hepatocellular carcinoma (HCC) is still one of the most common malignant tumors in the world [1] accounting for 75%– 85% of primary liver cancer cases. The onset of HCC is insidious, and it is not easy to be identified early. Most patients with HCC are in the middle and late stages at the time of diagnosis and lose the opportunity of surgical treatment, and the post-operative recurrence and metastasis rates are high. Being HCC a silent disease, the identification of a potential diagnostic biomarker is a thrust area of research in the field. Several biomarkers have been identified and introduced into clinical practice but insufficient specificity and sensitivity of those dictate the necessity for novel peptide discovery [2]. The identification, standardization, and validation of effective tumor biomarkers can dramatically influence cancer diagnosis, prognosis, and anti-cancer drug development.

Functional and morphological changes of hepatocytes, sinusoidal endothelium, Kupffer cells, and stellate cells during HCC impair normal liver blood supply systems and thereby cause tissue hypoxia [3–5]. Moreover, the HCCs are also typically accompanied by neovascularization and hypervascularity [6–8]. So, hypoxia is believed to participate in the genesis and progression of HCCs and the biomarkers involved in this process can also play an important role.

Adrenomedullin (AM), a member of the calcitonin peptide superfamily which also included calcitonin, calcitonin gene-related peptide, amylin, and intermedin, appears to be a new metastasis-related factor involved in liver cancer [9]. The peptide was first isolated from pheochromocytoma, but it was later found to be expressed by many body tissues and to play a key role as a mediator of inflammation via paracrine, autocrine, and endocrine mechanisms [10]. Later, it was speculated that AM might be involved in tumor progression by promoting proliferation and inhibiting apoptosis through several signaling pathways [11–13], and also by acting as an angiogenic factor [11–15].

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AM and its family peptides act via a G protein-coupled, seven-transmembrane domain receptor, calcitonin receptor-like receptor (CLR) [16]. CLRs associate with an accessory protein, receptor activity-modifying protein (RAMP), which comprises about 160 amino acids and includes a single membrane-spanning domain. Three RAMP isoforms (RAMP1, 2, 3) have been identified. When interacting with RAMP1, CLR exhibits a high affinity for calcitonin gene-related peptide, and when interacting with either RAMP2 or 3, CLR exhibits a high affinity for AM [17]. Anyway, RAMP2 is known to be not expressed in human hepatic tissue [18]. AM transcript has been demonstrated in several cancer cell lines, such as brain tumors [19] neuroblastoma [20], several lung cancer cell lines [21], and pancreatic cancer [22]. In addition, the AM and its receptor expression have been demonstrated in many human epithelial cancer cell lines of diverse origins (e.g., lung, colon, ovary, breast, bone marrow, prostate, and cartilage cell lines [23]). It was also demonstrated that AM signaling is hypoxia-inducible and functionally active in HCCs; on the other hand, hypoxia is a common characteristic of many solid tumors and is associated with malignant progression, distant metastasis, and resistance to treatment [24].

However, the AM role has not yet been extensively explored during HCC progression as well as in extracellular vesicles (EVs) secreted by HCC tumor cell lines. Over the last decade, the field of EVs has gained prime importance with the discovery of their role as novel mediators in cell signalling. They can be divided into three categories: exosomes, microvesicles, and apoptotic bodies and are presenting in all biological fluids playing pleiotropic roles in cell-to-cell communications [25,26]. Emerging studies have shown that EVs are very important in regulating the proliferation, immune escape, and metastasis of HCC [27]. They carry various cargoes of proteins, lipids, and genetic materials that can deliver signals to induce physiological or pathological changes in recipient cells [28] but at present, data on AM expression levels in EVs are missing.

The study aimed to evaluate the transcriptional profile of AM and its specific receptors CLR, RAMP1, RAMP3 in liver tissues of subjects with hepatitis C virus (HCV)-positive HCC undergoing liver transplantation and in donors. Moreover, given the important role of the EVs as novel mediators in cell signalling, we also evaluated whether AM and its receptors are carried by EVs secreted by tumorogenic (HepG2) and by non-tumorigenic (WRL68) cells.

2. Material and methods

2.1. Patients selection and sample collection

Twenty-eight subjects (n = 14 with HCV- related HCC undergoing liver transplantation, liver recipients, LR, age: 59.4 ± 1.8 and n = 14 donor liver grafts, liver donors, LD, age: 62.1 ± 17.3) admitted for surgery to the liver transplantation unit of the University of Pisa, were enrolled. The same patients were studied by our group for the evaluation of osteopontin, osteoprotegerin, pentraxin, and apelin as possible biomarkers for diagnosis and follow-up of HCC [29–32]. Four patients with HCV- related HCC had diabetes, and for consistency of results were excluded from the analysis. As reported in our previous studies [29–32] the exclusion criteria included acute liver failure, MELD scores 30 at transplantation, HCC beyond Milan criteria as per the pre-transplant clinical investigations, non-HCC liver neoplasms, bone metastasis, acute and/or chronic kidney disease, a body mass index (BMI)> 30 kg/m^2, combined organ transplantation, abstinence from alcohol use < 6 months, autoimmune liver disease on active steroid treatment, HCV-related disease on treatment with interferon at the time of transplantation, history of major cardiovascular event, such as acute myocardial infarction, unstable angina, previous coronary revascularization, stroke and peripheral vascular disease grade 2, hepato-pulmonary syndrome with arterial hypoxemia (PaO_2) < 70 mmHg or an alveolar-arterial gradient > 20 mmHg, porto-pulmonary hypertension with a mean pulmonary arterial pressure > 25 mmHg and mental impairment. While the plasma biochemical values of the donors were within the limits of normality, those of the recipients resulted out or upper limit of the normal range (AST: 109.3 ± 20.2 UI/L, ALT: 106.7 ± 19.5 UI/L, GGT: 110.5 ± 22.7 UI/L, Bilirubin: 1.16 ± 0.2 mg/dL, INR: 1.11 ± 0.04%, Creatinine: 1.2 ± 0.25 mg/dL). MELD score at transplantation resulted in 8.6 ± 0.6 (5 subjects with MELD score <9 and 5 subjects with MELD score between 10 and 13). The subjects enrolled in this study not exceeded the MELD score of 13. Approval was obtained from the Institutional Ethics Committee. The study was carried out in compliance with the principles set forth in the 2008 Seoul revision of the declaration of Helsinki.

2.2. Cell culture and isolation of EVs by differential centrifugation

As reported in a previous study of ours [33] the human HepG2 HCC cell line (Sigma-Aldrich, St. Louis, MO, USA) and the human WRL68 normal hepatocyte cell line (Sigma-Aldrich) were cultured in Eagle's Minimal Essential Medium (EMEM) (Sigma-Aldrich) containing 1% non-essential amino acids, 10% fetal calf serum (FCS) (Life Technologies, Carlsbad, CA) and penicillin/streptomycin. The medium was renewed every second day.

For the experiments, cells were passaged in T-150 culture flasks (Corning, NY, USA) and cultured at half-confluence. Seventy-two hours before the supernatant collection, monolayers were washed with EMEM without FCS, and the medium was replaced with EMEM containing EV-depleted FCS (Life Technologies).

As previously reported [33] the EVs were isolated by the supernatant of each cell line, through differential centrifugation. Briefly, a culture medium by 72 h of incubation was collected (120 mL) and centrifuged at 480xg to remove floating cells and cell debris. Pellet was discarded and the supernatant was centrifuged at 2000xg for 30 min to separate apoptotic blebs. The supernatant was filtrated using 0.22-μm filters and ultra-centrifuged at 100,000xg (Beckman coulter optima LE-80 K ultracentrifuge and 50.2 Ti Rotor, CA, USA) for 2 h at 4 °C to obtain EVs. Analysis of optical microscopy images does not support the presence of HeLa cells in our cell samples.

2.3. RNA extraction

HCC tissue samples: hepatic samples were collected in the course of the surgical procedure from LD and LR and preserved in RNA*later* (Sigma-Aldrich, St. Louis, MO, USA) at − 20 °C as previously reported [29–32]. Total RNA was extracted from liver tissue samples with Rneasy Midi kit (Qiagen S.p.A, Milano, Italy) and stored at − 80 °C for use in Real-Time PCR studies.

EVs isolated by HepG2 and WRL68 cell culture: RNA extraction was carried out using acid guanidinium thiocyanate– phenol-chloroform form (Qiazol, Qiagen SpA, Milano, Italy) following miRNeasy Mini kit manufacturer's instruction (Qiagen SpA, Milano, Italy) as also reported in a previous study of ours [33]. After resuspension and lysis of the EVs with insulin syringes to break the cell membrane and to allow acid guanidinium thiocyanate-phenol-chloroform to enter at intracellular level, samples were selectively bound on a silica-based membrane and speeded on a micro spin centrifuge. A specific high-salt buffer system allows RNA to bind to the miRNeasy silica membrane and contaminants were washed out. High-quality RNA was then eluted in 15–30 μl of RNAse-free water [33]. 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.

2.4. Transcriptional analysis

In all types of samples, the transcriptional analysis of the AM system was performed by Real-Time PCR (CFX-96 Real-Time PCR detection systems, Bio-Rad).

HCC tissue samples: cDNA was produced with iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA) and preserved at 4 °C until Real-Time PCR reactions were executed. As previously described the amplifications were performed in duplicate in the Bio-Rad C1000 TM thermal cycler (Bio-Rad) [29–32]. Apoptotic markers, as Caspases-3, Bcl-2, BAX, and NOTCH-1 were also measured.

EVs isolated by HepG2 and WRL68 cell culture: Total RNA extracted from the EVs was reverse transcribed with miScript II RT Kit (Qiagen S.p.a., Milano, Italy). The cDNA samples obtained were stored at 4 °C until Real-Time PCR analysis was performed as for HCC tissue samples [33].

For all samples the cDNA amplification reaction was monitored with a fluorogenic DNA binding dye, EvaGreen (SsoFAST Eva Green Supermix Bio- Rad Laboratories Inc., CA) and the optimal Real-Time PCR conditions were developed for each gene analyzed. The efficiency and the linearity of amplification were evaluated varying primer annealing temperature and sample concentration. The standard curve was obtained by sample scalar dilution from 1:5–1:625 and the efficiency ranged from 95% to 105% with a R^2 coefficient $\geq$ 0.998. In order to verify the specificity of the amplification products, the amplicons were tested through melting curves analysis. Since Real-Time PCR efficiency is highly dependent on the primers used, their sequences were accurately selected and whenever possible, intron-spanning primers were selected to avoid amplification of genomic DNA. To better improve primers specificity, the regions of homology were checked and eluded as well as secondary structures leading to poor or no yield of the product was avoided. The MIQE (Minimum Information for publication of Quantitative Real-Time PCR Experiments) guidelines [34] were followed and the primer pairs used for Real-Time PCR study were designed with Primer Express Version 2.0 (Applied Biosystems) and/or with a specific software Beacon Designer® (version 8.1; Premier Biosoft International, PaloAlto, CA) concerning nucleotide sequences included in the NCBI database GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) (Table 1). All primers were synthesized by Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

2.5. Statistical analysis

Both for the experiments carried out in HCC tissue samples than in EVs isolated by HepG2 and WRL68 cell culture, the geometric mean of the three most stably expressed genes was used for normalization of Real-Time PCR results. As previously tested [29–32] for HCC tissue samples they resulted to be PPIA, eEF1a, TPT1 (M value=0.69) while for EVs isolated by HepG2 and WRL68 cell culture they resulted to be eEF1a, RPL13a, RPS4X (M=0.79). The relative quantification was obtained by the ΔΔCt method using Bio-Rad's CFX96 manager software. When mRNA values resulted not normally distributed the statistical analysis was conducted after logarithmic transformation of data. Fisher's test after ANOVA and unpaired t-test were used to obtain the results that were expressed as mean ± SEM; $p < 0.05$ was considered significant. The association between different variables was assessed by linear regression test after logarithmic transformation, when necessary. All data were analysed by using Statview 5.0.1 software released for Windows Statistical (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Gene expression profile

HCC tissue samples

The Adrenomedullin mRNA expression resulted significantly higher in the recipient's liver samples with respect to donors (Fig. 1a) while as for mRNA receptor expression we did not observe any significant difference between the two analysed groups, even if RAMP3 and CLR showed higher levels in LR with respect to LD (Fig. 1b-d). No regression was observed between AM and its receptors expression levels while a significant correlation was found between CLR and RAMP1 ($r = 0.52$, $p = 0.01$). When LR patients were divided into groups according to the MELD score, we observed significantly higher levels of AM expression in patients with MELD score < 9 and with MELD score between 10 and 13 compared to LD patients (Fig. 2a). mRNA receptor expression levels of

Table 1
Primer sequence details of the analyzed gene.

GENES	*PRIMER SEQUENCE*	*GENBANK, ACCESSION N.*	*LENGTH (bp)*	*TEMP (°C)*
eEF1a	**F:** CTTTGGGTCGCTTTGCTGTT **R:** CCGTTCTTCCACCACTGATT	NM_001402	183	60
PPIA	**F:** CTTGGGCCGCGTCTCCTTCG **R:** TTGGGAACCGTTTGTGTTTGGGGC	NM_021130	285	60
TPT1	**F:** AAATGTTAACAAATGTGGCAATT **R:** AACAATGCCTCCACTCCAAA	NM_003295	164	60
RPL13a	**F:** CGCCCTACGACAAGAAAAAG **R:** CCGTAGCCTCATGAGCTGTT	NM_012423	206	60
RPS4X	**F:** GATCCCCTCATCAAGGTGAA **R:** GCCCTTGCCAATAACAAAAA	NM_002046	243	60
AM	**F:** GCAAGCCTCACTATTACT **R:** ATTGATAGTTACCTCTGTATGT	NM_001124	84	60
RAMP1	**F:** GAGGTGGACAGGTTCTTC **R:** ATGAAGGGGTAGAGGATG	NM_005855	107	60
RAMP3	**F:** TGGAGGAAAATGTGATAAG **R:** AGAAACAGCATAGAAACC	NM_005856	116	60
CLR	**F:** CCTGATGTGACGCTACTAACCTGAC **R:** AATGGTGTGCTGGAACTGGCTTA	NM_005795	100	60
Bcl-2	**F:** CCGACCACTAATTGCCAAG **R:** TTCCATCCGTCTGCTCTT	NM_000633	121	58
BAX	**F:** ACCAGGGTGGTTGGGTGAGACTC **R:** TCCAGGGAGGGCAGAAGGCACTA	AY217036.1	289	58
NOTCH-1	**F:** AGAACTGTGAGGAAAATATCG **R:** TACTGACCTGTCCACTCT	AF308602	118	60
CASP-3	**F:** CTGTAACTTGAGAGTAGATGGT **R:** ATGGAGAAATGGGCTGTAG	NM_032991	110	60

eEF1a: Eukaryotic translation elongation factor 1 alpha 1; ***PPIA***: Peptidylpropyl isomerase A [cyclophillin A]; ***TPT1***: Tumor protein, translationally-controlled 1; **RPL13a**: ribosomal protein L13a; **RPS4X**: ribosomal protein S4 X-linked; ***AM***: adrenomedullin; ***RAMP1***: Receptor activity modifying protein 1; ***RAMP3***: Receptor activity modifying protein 3; **CLR**: calcitonin receptor like receptor; ***Bcl2***: beta-cell leukaemia 2; ***BAX***: bcl-2-like protein 4; ***NOTCH-1***: Neurogenic locus notch homolog protein 1: **CASP-3**: caspase 3.

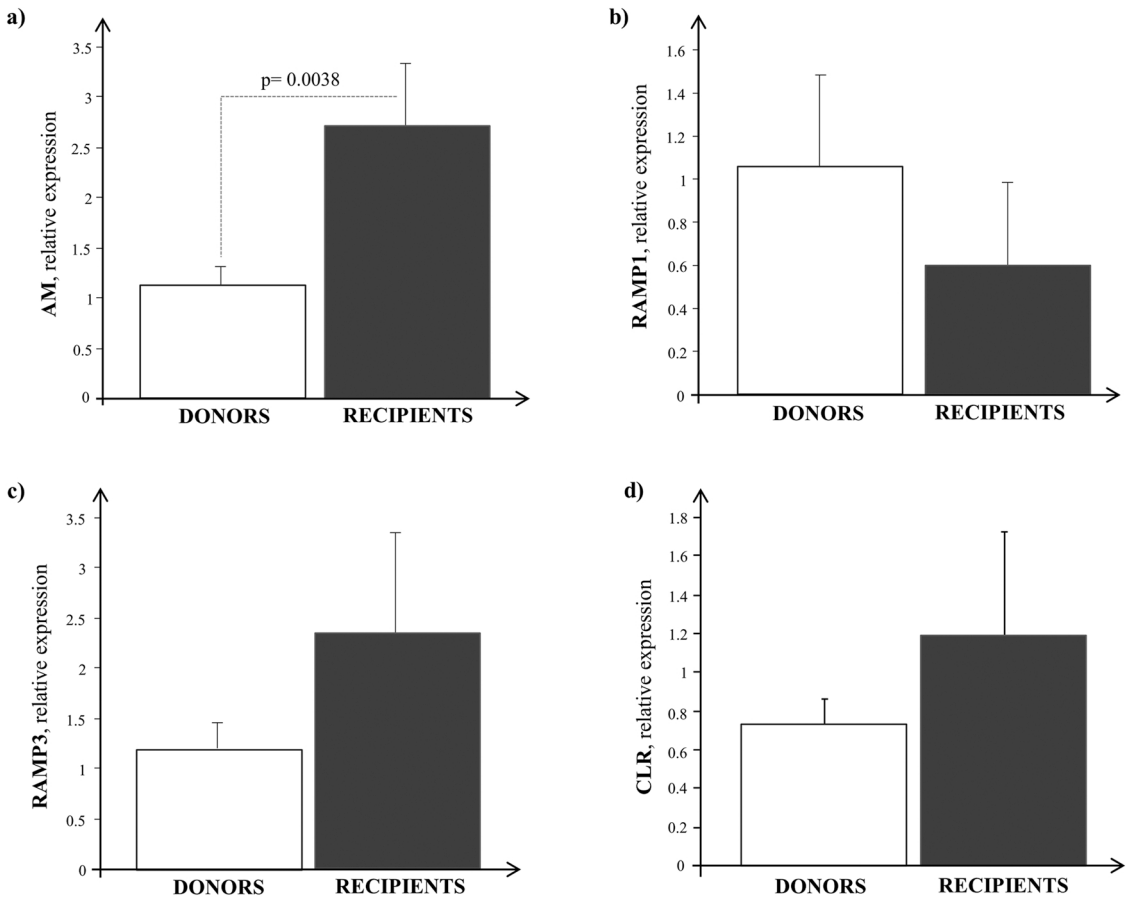

Fig. 1. a) Adrenomedullin, b) RAMP1, c) RAMP3, d) CLR mRNA expression in donor and recipient's liver samples (white and dark grey bar).

RAMP3 and CLR increased in patients with HCC as a function of clinical severity while RAMP1 levels decrease as a function of clinical severity (Fig. 2b-d).

The mRNA expression of Bcl-2, BAX, NOTCH-1, and Caspase-3 was evaluated by us in the same group of patients of this study and the results were previously reported [31]. In brief, mRNA expression resulted higher in recipient's liver samples with respect to donor samples, reaching levels of significance only for Bcl-2 ($p = 0.0029$). Moreover, the apoptotic markers increased as a function of HCC severity but with a significant difference only for NOTCH-1 and Bcl-2, which featured significantly higher levels in LR with MELD between 10 and 13 with respect to donor samples [31]. Positive correlations were also found between AM mRNA expression and NOTCH-1 ($r = 0.50$ $p = 0.01$), BAX ($r = 0.45$ p p $= 0.003$) as well as between RAMP3 and BAX ($r = 0.62$ $p = 0.0017$) and NOTCH-1 ($r = 0.57$ $p = 0.0042$) while a negative correlation was found between RAMP1 and Caspase-3 ($r = 0.42$ $p = 0.05$). Bcl-2/BAX ratio, evaluated in function of MELD score, showed a similar trend with respect to AM mRNA expression resulting higher in patients with MELD score < 9 (4.22 ± 2.12 relative expression) rather than those with MELD between 10 and 13 (3.00 ± 1.23 relative expression).

EVs isolated by HepG2 and WRL68 cell culture.

No difference for AM mRNA expression was observed between EVs isolated by tumorigenic hepatocyte cell line in comparison to non-tumorigenic hepatocyte cell line (Fig. 3a). mRNA expression of both RAMP1 and RAMP3 resulted lower in HepG2 with respect to WRL68 (Fig. 3b-c), while significant higher levels were observed for CLR mRNA expression. Moreover, we observed a significative positive correlation between AM and RAMP3 ($r = 0.66$, $p = 0.02$) and a significative negative correlation between CLR and RAMP1 ($r = 0.69$, $p = 0.01$).

4. Discussion

In this study, the transcriptional profile of AM and its receptors was studied in HCC tissue samples and in EVs isolated from tumorigenic and non-tumorigenic hepatocyte cell lines demonstrating the presence of their expression in all samples analysed.

The AM mRNA levels resulted significantly elevated in liver tissues of subjects with HCV-positive HCC undergoing liver transplantation with respect to donors in line with previous studies where AM and HCC were analyzed [9,22,24] and, for the first time, higher levels in HCC patients were observed as a function of clinical severity. In fact, dividing the patients into groups according to the MELD score we observed higher levels of AM expression both in patients with MELD$<$ 9 and with MELD between 10 and 13 with respect to LD patients, confirming a role for AM in the progression of HCC. The trend of the lowest expression levels in LR compared to LD observed for RAMP1 and the trend of the highest levels of RAMP3 and CLR observed in LR compared to LD was confirmed by analysing them according to the severity of the disease.

The increase in the amount of AM expression in cancerous tissues could be positively associated with metastasis and tumor progression and could be explained by the regulation of some factors involved in tumor initiation and metastasis as for example the hypoxia. Hypoxia is a common characteristic of many solid tumors and is associated with malignant progression, distant metastasis, and resistance to treatment [35]. Recently, was observed that hypoxia participates in the genesis and progression of HCC [36–39] and that adrenomedullin signaling is hypoxia-inducible and functionally active in HCC accelerating tumor cell growth [24].

Moreover, it is known that AM may also induce angiogenesis [40] and the higher levels of AM mRNA expression found in this study as a function of MELD severity, could suggest an important role for this

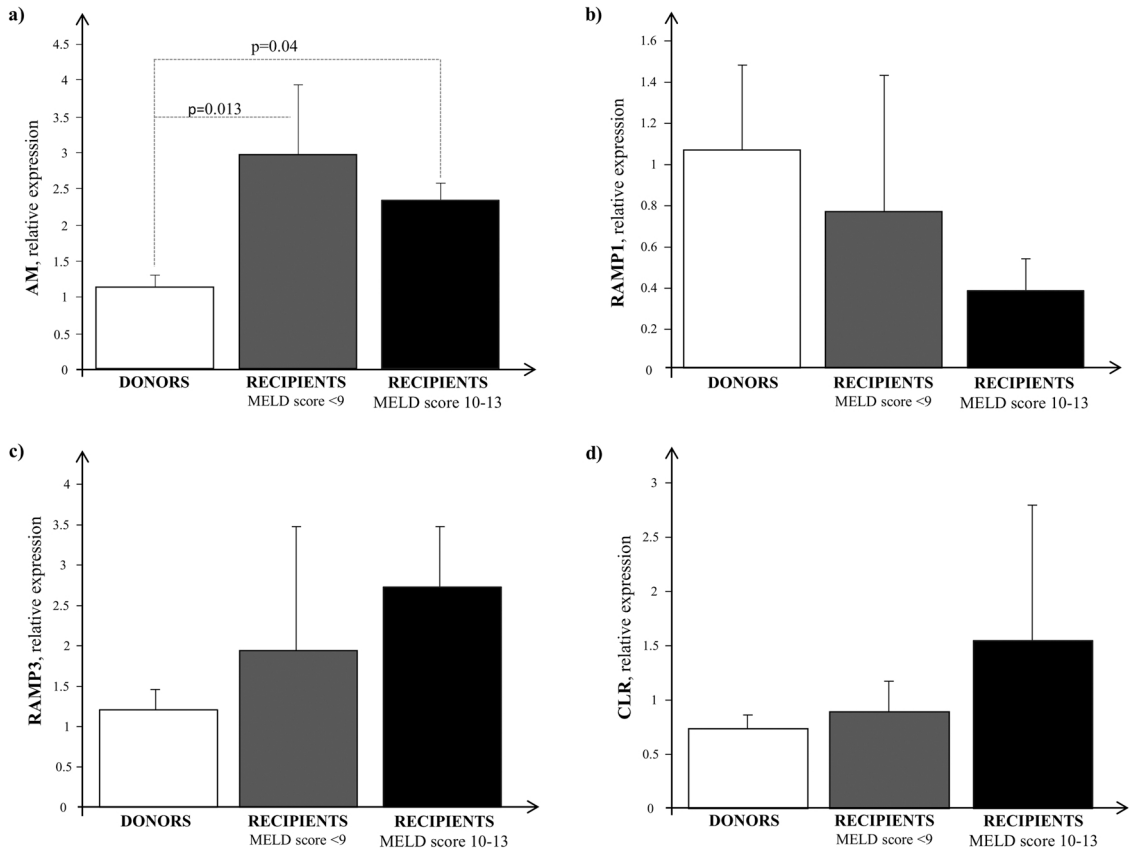

Fig. 2. a) Adrenomedullin, b) RAMP1, c) RAMP3, d) CLR mRNA expression in HCC patients as a function of clinical severity using MELD score (donors: white bar; recipients-MELD score<9: dark grey bar; recipients-MELD score 10–13: black bar).

biomarker during cancerogenesis even if the mechanism by which AM expression increased in HCC remains unclear.

Our results also confirmed a possible correlation between AM and tumor cell apoptosis and, as literature data reported, the inhibition of apoptosis during tumorigenesis may play a crucial role in the development and progression of different cancers [41,42]. So these results could provide important insights into the role of AM during HCC.

On the other hand, while the variations in the AM levels expression, observed as a function of the disease in HCC subjects, could suggest important indications on the progression of the disease, the study of the AM expression levels in EVs secreted by hepatic tumorigenic cells is not able to provide new potential tools for the diagnosis and development of HCC. So, while for many other biomarkers [25,27,28,33] the study of their expression in EVs secreted by hepatic tumorigenic cells can play an important role as a potential new therapeutic tool/target in cell signalling during HCC, the AM involvement in this model is not probably rather relevant and the study of its expression is not able to predict therapeutic outcomes and design of more effective personalized cancer treatment plans.

5. Conclusions

We can conclude that while the results obtained about AM mRNA tissue levels as a function of clinical severity could characterize the AM as a regulator of carcinogenesis-tumor progression, identifying it as a putative target for developing new strategies against liver cancers, those obtained in EVs do not indicate the AM as a target candidate neither as pathological biomarker nor as marker involved in cancer therapy.

Ethics approval and Consent to participate

The study was carried out in compliance with the principles set forth in the 2008 Seoul revision of the declaration of Helsinki. Approval was obtained from the Institutional Ethics Committee.

All patients enrolled gave their consent to be included in the study.

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CRediT authorship contribution statement

All authors contributed to the study conception and design. Conceptualization, data curation, formal analysis: Silvia Del Ry. Methodology: Silvia Del Ry, Manuela Cabiati, Melania Gaggini Costanza Salvadori, Serena Del Turco, Chiara Caselli, Antonella Cecchettini. Patient enrolment: Paolo De Simone. Writing – original draft: Silvia Del Ry. Writing – review & editing: Manuela Cabiati, Melania Gaggini, Serena Del Turco, Chiara Caselli, Antonella Cecchettini. All authors read and approved the final manuscript.

Conflicts of interest

There is no conflict of interest.

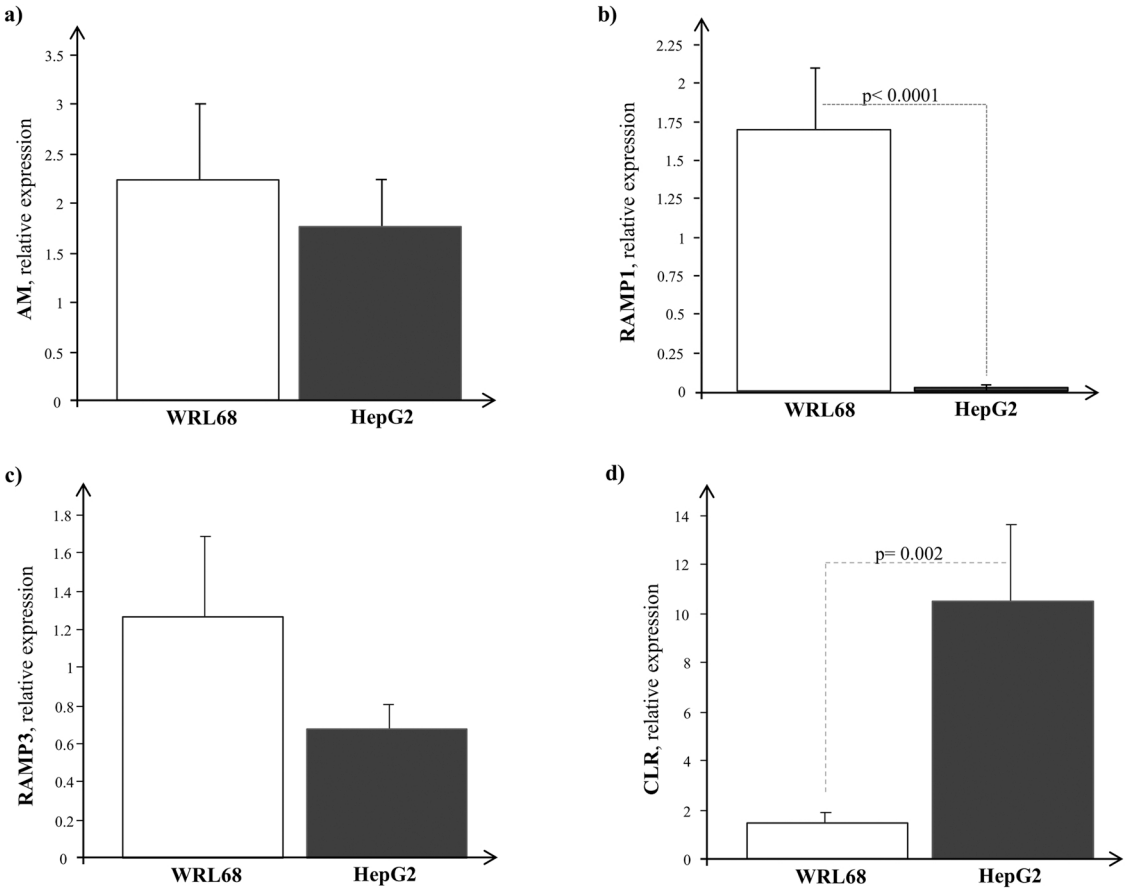

Fig. 3. a) Adrenomedullin, b) RAMP1, c) RAMP3, d) CLR in EVs secreted by tumorigenic (HepG2) and by non-tumorigenic (WRL68) cells (white and dark grey bar).

Consent for publication

All authors gave their consent for this paper publication.

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