

ORIGINAL ARTICLE

Augmenter of liver regeneration gene expression in human colon cancer cell lines and clinical tissue samples

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Summary

Purpose: Augmenter of liver regeneration (ALR) is an hepatotrophic factor responsible for the increased regenerative capacity of mammalian liver and ALR gene expression has been well-documented in liver cirrhosis and hepatocellular carcinoma tissue samples. The present study aimed to quantify and evaluate ALR gene expression in human colon cancer cell lines and tissue samples.

Methods: Total RNA was isolated from 6 colorectal cancer cell lines and 23 primary colorectal tumors, cDNA was prepared and ALR mRNA expression analysis was performed using quantitative real-time PCR.

Results: ALR mRNA expression was confirmed in all 6 colorectal cancer cell lines (SW480, SW620, DLD-1, RKO, COLO-205 and HTC-116) and an epithelial one (WISH). DLD-1 cell line showed the highest ALR mRNA levels, followed by RKO, COLO-205, HCT-116, SW480, SW620 and WISH cell lines. ALR gene expression levels were detected

in all cancer tissue samples (N=23), being significantly increased in well/moderately compared to poorly differentiated tumors ($p=0.0208$). ALR gene expression levels were increased in Dukes' stage A/B compared to stage C tumors, at a non significant level ($p=0.2842$). ALR mRNA levels were slightly higher in colon cancer tissues compared to adjacent non-neoplastic ones (N=19), at a non significant level ($p=0.2122$).

Conclusion: The present study verified for the first time the ALR gene expression in both human colon cancer cell lines and clinical samples. Enhanced ALR gene expression was negatively correlated with advanced histopathological grade and stage in both colon cancer cell lines and human tissue samples, implicating ALR participation at the early stage of colon malignant progression.

Key words: augmenter of liver regeneration, colon cancer cell lines, colorectal carcinoma, quantitative real-time PCR

Introduction

Colorectal cancer is the third most commonly diagnosed cancer and the cause of cancer-related deaths in the United States, when men and women are considered separately, and the second leading cause of cancer-related deaths when both sexes are combined [1]. More than 1 million individuals worldwide will develop colon cancer every year [2]. The incidence rates are tenfold higher in developed than in developing countries [3]. Colorec-

tal cancer is treatable if detected and surgically removed at an early stage with 95% of patients surviving beyond 5 years [4].

In 1975, La Brecque and Pesch first [5] and later Fleig et al. [6] referred to the existence of a polypeptide, in the liver cytosol of weanling or adult partially hepatectomized rats, named hepatic stimulator substance (HSS). HSS was able to specifically stimulate hepatocyte proliferation and support liver regeneration in an organ-specific but species non-specific manner [5,6]. Since

then, a number of *in vivo* studies examined HSS levels in animal models of liver regeneration after partial hepatectomy (PH) or post-toxin induced injury [7-9]. A novel growth factor, obtained through progressive purification of the crude HSS extract, was named Augmenter of Liver Regeneration (ALR) [10,11]. Different studies verified that ALR, as well as HSS, did not affect quiescent hepatocytes but enhanced their proliferation in response to PH in rats [12,13] and dogs [14] and also prevented atrophy increased cell renewal caused by portacaval shunt (Eck's fistula) in dogs [15,16]. Although ALR has been initially described as an hepatic growth factor promoting regeneration, it is not restricted to liver only. More recently, ALR was found to be ubiquitously expressed in several tissues with highest detected expression levels in liver and testis and lower in muscle [17,18].

Human, mouse and rat ALR gene and cDNA have been cloned and the complete sequences are already known [18-21]. The human ALR gene is located on chromosome 16, at the cytogenetic band 16p13.3-p13.12, in the interval containing the locus for polycystic kidney disease (PKD1) [22] and encodes the ALR protein (Alrp). Alternative splicing generates two forms of Alrp: the shorter protein, consisting of 125 amino acids (15 kDa) and lacking the amino terminus 80 amino acids and the longer protein with 205 amino acids (23 kDa) [18,23]. The three dimensional structures of Alrp have also been examined [24]. The carboxy terminus of the human Alrp was characterized as structural and functional homologue for yeast Erv1p [22]. Alrp and yeast Erv1p belong to a protein family, members of which are found in lower and higher eukaryotes from yeast to man [19,20,22,25] and even on the genome of some double stranded DNA viruses [26-29]. Homologous proteins were also found in plants [30]. They have essential functions in the biogenesis of mitochondria, the cell division cycle and in the development of organs such as liver and testis in higher eukaryotes [31]. Moreover, Alrp/Erv1p family belongs to flavin-linked sulfhydryl oxidase participating in disulfide bond formation [32,33].

Although the expression of ALR gene has been examined in liver with chronic diseases (cirrhosis and hepatocellular carcinoma/HCC) and cholangiocarcinoma (CCC), there are no reports to indicate its presence in colon. In this aspect, the present study aimed to investigate, for the first time, ALR gene expression in human colorectal cell lines, as well as in colon cancer clinical samples and to correlate it with clinicopathological

parameters important for patients' management and prognosis.

Methods

Cell lines

Colorectal cell lines (SW480, SW620, SLDL-1, RKO, COLO 205 and HCT 116) and non-colorectal cell line (WISH), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The 6 corrected cancer lines were classified according to the stage of tumor from which they were established: SW480 as Dukes'stage B; SW620 and DLD-1 as Dukes'stage C; COLO 205, HCT116 and RKO as Dukes'stage D. SW480 and SW620 were cultured in ATCC-formulated Leibovitz's L-15 medium (GIBCO Life Technologies Inc, Gaithersburg, MD), DLD-1 and COLO 205 in ATCC-formulated RPMI-1640 medium (GIBCO Life Technologies Inc, Gaithersburg, MD), whereas, HCT 116 in ATCC-formulated McCoy's 5A medium (GIBCO Life Technologies Inc, Gaithersburg, MD), RKO and WISH in ATCC-formulated Eagle's Minimum Essential Medium (GIBCO Life Technologies Inc, Gaithersburg, MD). All cell lines were supplemented with 10% fetal bovine serum (FBS) (GIBCO Life Technologies Inc, Gaithersburg, MD), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO Life Technologies Inc, Gaithersburg, MD). All cells were incubated at 37°C in a humidified incubator with 5% CO₂, except for SW480 and SW620 cell lines in which CO₂ is not necessary. When cells reached 80% confluence, they were dissociated in 0.5% trypsin and 0.2 g/mL EDTA (GIBCO Life Technologies Inc, Gaithersburg, MD).

Clinical material

Twenty three patients with colon cancer operated from 2007 to 2009 in the Second Department of Propeedeutic Surgery, Medical School of National and Kapodistrian University of Athens, constituted the study group. The study was approved by the ethical committee of "Laikon" General Hospital. Eleven (48%) patients were men and 12 (52%) women with a mean age of 68.8 ± 10 years (range 51-86). Tumors were classified and staged according to the revised guidelines set by American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC) [34,35]. Three levels of differentiation were used to clarify histopathological grading: (a) well in 6 cases (26%), (b) moderately in 12 (52%) and (c) poorly in 5 (22%), respectively. The resected tumors were histologically staged according to Dukes' classification as: A in 4 cases (17%); B in 10 cases (43%) and C in 9 cases (39%). Nineteen of the fresh tissue specimens were analyzed in conjunction with the matching non-neoplastic tissue samples. The selection criteria for the above samples included availability of sufficient tissue mass for RNA extraction and assay.

Total RNA extraction

Colon tissues were snapped frozen in liquid nitrogen and after homogenization of specimens in TRI Reagent (Molecular Research Center Inc., Cincinnati, USA). Following the manufacturer's instructions, total RNA was extracted from frozen tissue biopsies and cell lines using NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany) and then stored at -80°C. The RNA concentration was determined using a UV/VIS spectrophotometer (Pharmacia Ultrospec, Cambridge, UK) and standardised to 40 ng/μL by dilution with sterile DNA-free DEPC-treated water.

cDNA synthesis

Total RNA was reversed-transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) in accordance with manufacturer's instructions.

Quantitative real - time PCR

Quantitative real-time PCR was developed for the quantification of mRNA expression levels of ALR and c-Abl (as internal control or housekeeping gene), using the 1.5 LightCycler instrument (Roche, Penzberg, Germany) according to the manufacturer's instructions. In each quantitative real - time PCR reaction we used for ALR forward primer 5'-GGGACACCAAGTTTAGGGA-3' and reverse 5'-TCCTGCACAGCCTTTTTC-3' (TIB MolBiol, Berlin, Germany) producing a 206 bp amplicon and for Abl forward primer 5'-TTCAGCGCCAGTAGCATCTGACTT-3' and reverse 5'-GACCCGGAGCTTTTACCTTTAGTT-3' producing a 185 bp amplicon [36,37]. Reactions were performed in a 20 μl volume with 0.5 nM primers and 1.0 ng/μl cDNA and Light-Cycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The reaction conditions were denaturation at 95°C for 10 min, followed by 40 cycles with a 95°C denaturing for 10 sec, annealing at 61°C for 10 sec and extension at 72°C for 15 sec. A final cycle was performed to construct a dissociation curve in order to distinguish the main PCR product from primer dimmers and/or other non-specific products. Melting curve analysis of the PCR products was generated after amplification, by heating reaction mixture from 56°C to 95°C with a heating rate of 0.1°C/sec and continuously accurate fluorescence emission data. Comparative quantitative determination of expression levels for all genes was realized with application of the $\Delta\Delta C_t$ method [38]. Normalization of results for all genes became as for internal marker (housekeeping gene, *GAPDH*) and as representing normal sample. All specimens were assayed in triplicate.

Statistics

Kolmogorov-Smirnov test was initially applied to assess the normality of ALR expression levels distribution. ALR expression levels were not normally dis-

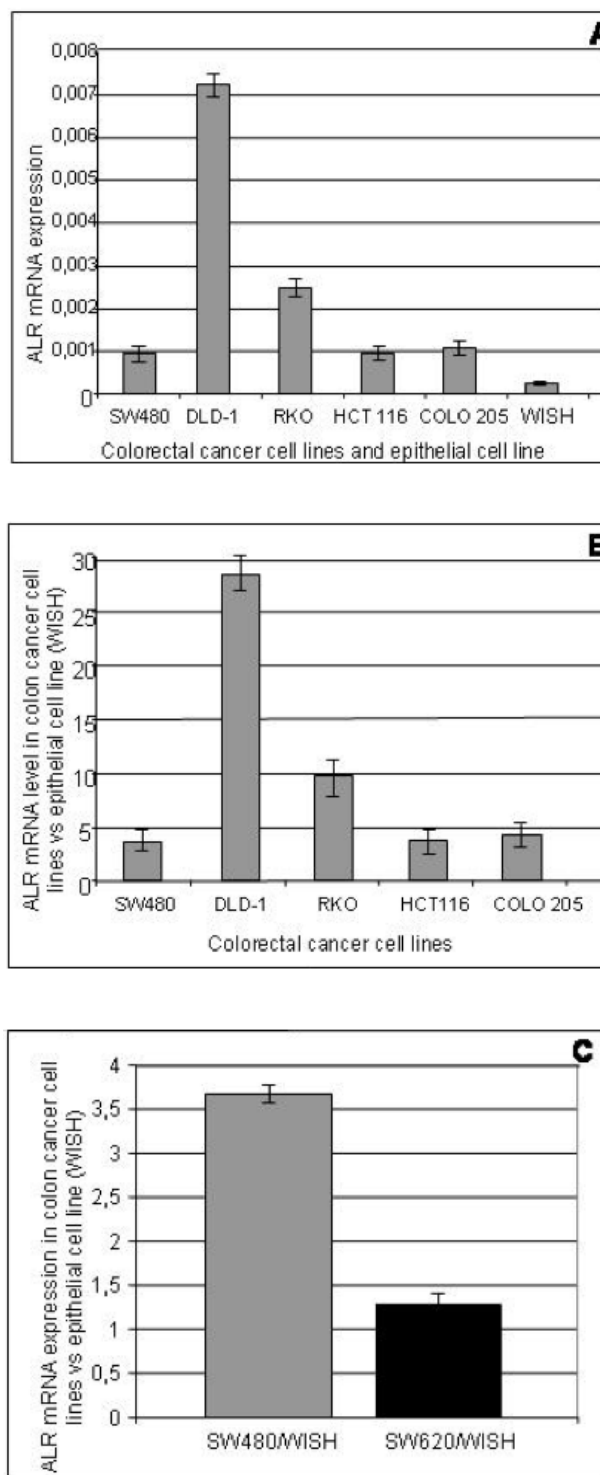


Figure 1. A: ALR mRNA expression levels in colorectal cancer cell lines (SW480, DLD-1, RKO, HCT 116 and COLO 205) and an epithelial one (WISH). Values are expressed as mean \pm S.E.M. **B:** ALR mRNA expression levels in colorectal cancer cell lines (SW480, DLD-1, RKO, HCT 116 and COLO 205) vs an epithelial one (WISH). Values are expressed as mean \pm S.E.M. **C:** ALR mRNA expression levels in the primary colon adenocarcinoma SW480 cell line (classified as Dukes' stage B) cell line compared to the levels observed in the metastatic colon SW620 cell line (classified as Dukes' stage C). Values are expressed as mean \pm S.E.M.

tributed and therefore non-parametric methods were used to assess the association of ALR expression levels with patients' age and gender, tumor histopathological grade and stage and tumor proliferative capacity (Mann-Whitney U test). Wilcoxon matched pairs test was used to assess the difference between the expression levels of cancer and adjunct matching non-neoplastic tissue in the examined cases. Values are expressed as mean \pm standard error of the mean (S.E.M.). Statistical analyses were performed using the software package SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA).

Results

ALR gene expression in colorectal cancer cell lines

ALR mRNA expression was detected in 6 colorectal cancer cell lines (SW480, SW620, DLD-1, RKO, HCT 116, COLO 205) and in an epithelial cell line (WISH) by quantitative real-time PCR. All colorectal cancer cell lines showed higher ALR gene expression levels compared to the epithelial one (Figure 1A,1B). The highest ALR gene expression levels were noted in the DLD-1 cancer cell line, followed by the RKO cell line. HCT 116, COLO 205 and SW480 cancer cell lines showed almost similar ALR gene expression levels (Figure 1A,1B). ALR mRNA levels were increased in the primary colon adenocarcinoma SW480 (classified as Dukes' stage B) cell line compared to the levels observed in the metastatic colon SW620 (classified as Dukes' stage C) cell line (Figure 1C).

ALR gene expression profile in human colon clinical samples in association with tumor histopathological grade and stage

Twenty-three primary colon cancer tissue samples were subjected to analysis. ALR gene expression levels in cancer tissue samples were significantly increased in well/moderately well compared to poorly differentiated tumors (Figure 2A, Mann-Whitney U test, $p=0.0208$). ALR gene expression levels in cancer tissue samples were increased in Dukes' stage A and B tumors compared to Dukes' stage C, at a non significant level though (Figure 2B, Mann-Whitney U test, $p=0.2842$). No significant associations or trends of correlation between ALR gene expression levels in cancer tissue samples and patients' age, gender and tumor proliferative capacity were noted (data not shown).

ALR expression in human colon cancer tissue versus normal, matching, non-neoplastic tissue

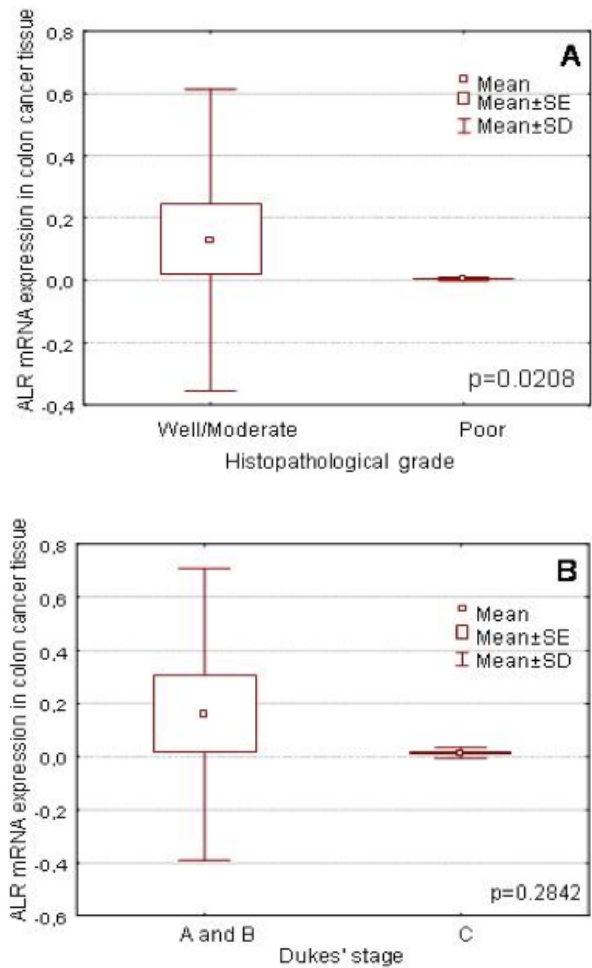


Figure 2. A: ALR mRNA expression levels in colon cancer tissue samples in relation with tumor histopathological grade of differentiation. **B:** ALR mRNA expression levels in colon cancer tissue samples in relation with Dukes' stage.

Nineteen primary colon cancer tissue samples were compared with the matching, non-neoplastic tissue. The expression level of ALR gene in cancer tissue samples was slightly higher compared to those of normal, matching, non-neoplastic ones, at a non significant level though (Figure 3A, $N=19$, Wilcoxon matched pairs test, $p=0.2122$).

The ratio of ALR gene expression in cancer vs normal adjacent tissue was significantly increased in well/moderately well compared to poorly differentiated tumors (Figure 3B, Mann-Whitney U test, $p=0.0336$). The ratio of ALR gene expression in cancer vs normal tissue was increased in Dukes' stage A and B compared to Dukes' stage C tumors, at a non significant level though (Figure 3C, Mann-Whitney U test, $p=0.1281$). Non significant associations or trends of correlation between the ratio of ALR gene expression in cancer vs normal tissue and patients' age, gender and tumor proliferative capacity were noted (data not shown).

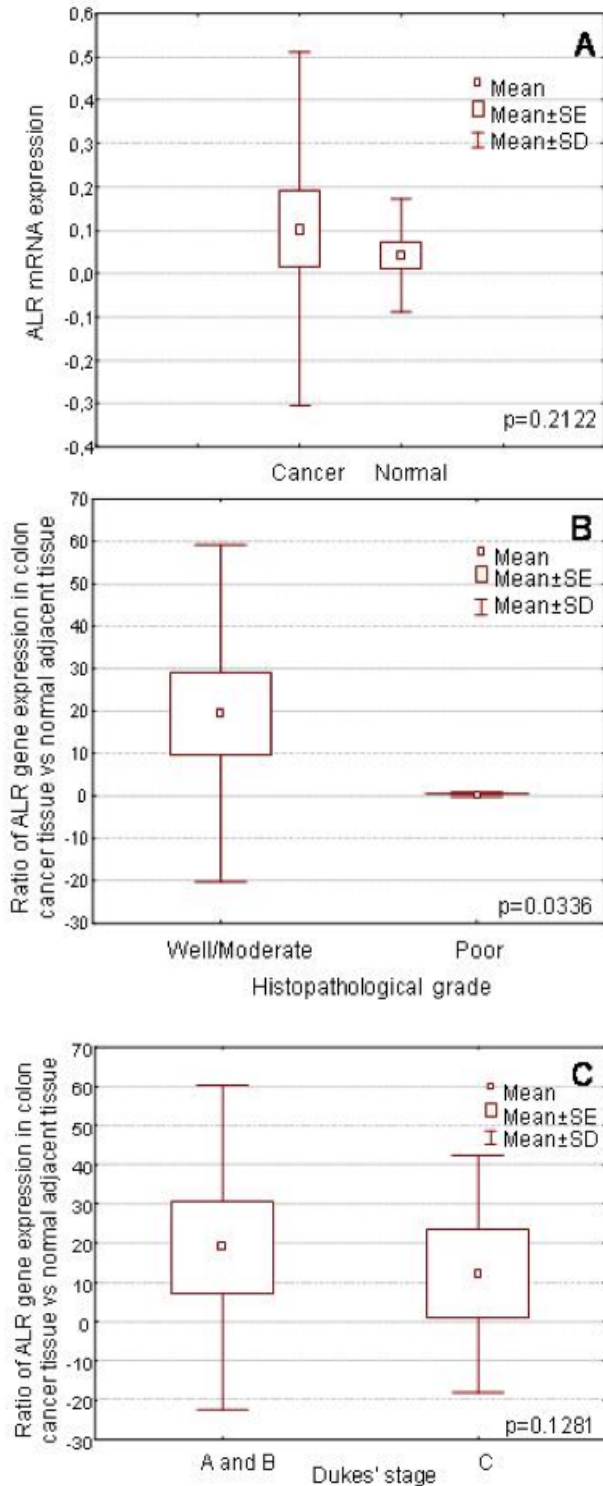


Figure 3. A: ALR mRNA expression levels in colon cancer tissue samples and normal, matching, non-neoplastic ones. **B:** ALR mRNA expression in cancer vs normal adjacent tissue in relation with tumor histopathological grade of differentiation. **C:** ALR mRNA expression in cancer vs normal adjacent tissue in relation with Dukes' stage.

Discussion

In the present study, ALR mRNA expression

was confirmed in all 6 colorectal cancer cell lines (SW480, SW620, DLD-1, RKO, COLO-205 and HTC-116) and one epithelial cell line (WISH). DLD-1 cell line showed the highest ALR mRNA levels, following RKO, COLO-205, HCT-116, SW480, SW620 and WISH cell lines.

The present study also showed that ALR gene expression was inversely related to the tumor histopathological grade and Dukes' stage. The enhanced ALR gene expression in well and moderately well compared to poorly differentiated tumors, as well as in Dukes' stage A and B compared to the more advanced Dukes' stage C tumors supported evidence for potential involvement of ALR gene at the early stage of malignant progression in the colon. Similarly, Dayoub et al., recently identified an inverse correlation of ALR expression with tumor histopathological grade, as also vascular invasion in HCC patients [39].

A matched pair of human primary and metastatic colon carcinoma cell lines, termed SW480 and SW620, was used as model system for identifying ALR gene expression with a potential role in metastasis. The SW480 and SW620 tumor cell lines established from the same patient have been previously characterized as primary and metastatic colon adenocarcinoma cell lines, respectively. The SW620 cell line was derived from a lymph node metastasis identified 6 months later during disease recurrence. Furthermore, both cell lines were isolated from the patient without prior chemotherapy. The SW480 cell line was classified as Dukes' stage B, while SW620 cell line as Dukes' stage C. Our results revealed that ALR mRNA levels decreased in SW620 cell line in comparison with the levels observed in SW480 cell line.

The present findings suggested that ALR mRNA levels were slightly higher in colon cancer tissues compared normal, matching, non-neoplastic tissues at a non significant level though. These data are in agreement with previous research in which ALR levels in HCC and CCC did not differ from the matching, non-neoplastic tissue [40].

As ALR was firstly identified in the liver, an increased interest in identifying its potential clinical value in the diagnosis of hepatic cirrhosis and HCC was noted. The first quantitative mRNA analysis revealed significantly increased ALR expression in cirrhosis, HCC and CCC compared with normal liver tissue [40]. These results indicated that ALR was regulated in the process of hepatocellular regeneration in liver cirrhosis and carcinogenesis [40]. Another study conducted on a larger sample population also documented that

ALR protein expression was augmented in HCC [39]. Although the methods used were not suitable to discriminate between different isoforms, it was shown that the expression of 15 kDa ALR in HCC cells attenuated hepatoma cell motility, supported and maintained epithelial cell growth both *in vivo* and *in vitro* and therefore, reversed the process of epithelial-mesenchymal transition [39].

In a recent study, liver ALR mRNA tissue and serum levels were measured in different liver diseases in order to determine the relationship between ALR, hepatocyte regeneration and disease state [41]. It was shown that different serum ALR levels were found in different hepatic failure patients, and they foreshowed completely different prognoses. Notably, patients with higher serum ALR levels had liver function improved enough through artificial liver support system therapy and rehabilitation to leave hospital [41]. Conversely, patients with lower ALR levels deteriorated to the point of death or had to receive orthotopic liver transplantation. Thus, it was speculated that decreased serum ALR levels may identify patients at risk of lethal liver failure and represent the trigger for maintaining intensive medical treatment or turning to salvage liver transplantation [41]. Moreover, it was shown that high serum ALR levels represented a high level of hepatocyte regeneration. The aforementioned survival rates of patients with different ALR levels were statistically significant, suggesting that ALR level may be helpful in estimating the survival time of patients with hepatic failure [41].

Under normal conditions, the longer isoform (23 kDa) of Alrp was mainly located in the cytosol and predominantly in the mitochondrial intermembrane space, where it participates in a chain of disulfide exchange reactions with Mia40 that generates disulfide bonds in a number of residence proteins with twin Cx3C and Cx9C motifs [42]. Moreover, CXXC motif of ALR is essential for cell survival and the biogenesis of cytosolic Fe/S proteins [43]. The shorter isoform (15 kDa) existed only in the nucleus and may act as an autocrine hepatotrophic growth factor promoting liver regeneration [44]. In this aspect, it has been suggested that ALR may augment liver regeneration via more than just one mechanism: suppression of liver natural killer cells, activation of MAPKs cascade in conjunction with regulation of EGFR expression and stimulation, regulation of NFkB and AP-1 activity [44].

Apoptosis is a hallmark of cancer cells. De-

fects in apoptosis can contribute to a variety of aggressive tumor phenotypes, including conferring an ability of tumor cells to survive after detachment from extracellular matrix and thus facilitating metastasis [45]. ALR exerts anti-apoptotic effects in human hepatocyte [46], neuroblastoma [47] and glioma cells [48] and peripheral blood lymphocytes [49]. The anti-apoptotic activity of ALR may be ascribed to the interaction of both intrinsic and extrinsic death pathways and apoptosis-related proteins [49]. As a consequence, the decreased levels of apoptosis in advanced stage tumor tissues may contribute to a reduction of ALR expression noted in our study concerning colon malignancy, as well as in the other available studies concerning HCC [39,40].

Conclusion

The present study provided for the first time preliminary evidence that ALR gene was expressed in human colon cancer cell lines and clinical tissue samples. An inverse relationship between ALR gene expression and tumor histopathological grade and stage was also recorded. This emerges a potential implication of the ALR gene at the early stage of colon malignant transformation. This data is in accordance to the fact that ALR gene expression was increased in human primary colon carcinoma cell line SW480 compared to metastatic SW620 one, both of which had been isolated from the same patient without prior chemotherapy. The present findings provide new insight for possible participation of ALR on the molecular mechanisms of colon tumor progression, reinforcing its potential utility to improve clinical therapy of colon cancer. It should be noted that, in the present research, the primers were designed to detect both splice variants. Thus, future research assessing the expression levels of the two transcripts of ALR that encode the two isoforms, may reveal their delicate and diverse functions in the fascinating and complex process of organ carcinogenesis. Moreover, this is a preliminary, pilot study that performed a quantitative analysis of the expression of the ALR gene. In this aspect, further studies conducted on larger population and including different types of human tumors and cancer cell lines are strongly recommended to confirm and extend the present findings and to facilitate the elucidation of the physiological and clinical significance of ALR in human malignancy.

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