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The role of PIAS3, p-STAT3 and ALR in colorectal cancer: new translational molecular features for an old disease

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Abstract. – OBJECTIVE: Human colorectal cancer (CRC) is characterized by a sequence of biological events that determine its induction and progression. Gut microbiota has an important role in this multistep model of carcinogenesis, as well as constitutive activation of Signal Transducer and Activator Factors 3 (p-STAT3) and Protein Inhibitor of Activated STAT3 (PIAS3), which negatively controls STAT3. It has been reported that a liver growth factor, the Augmenter of Liver Regeneration (ALR), an anti-apoptotic, anti-metastatic factor, exerts protective/cell survival and anti-metastatic activities and has been detected highly expressed in neoplastic cells.

PATIENTS AND METHODS: To evaluate, by immunohistochemistry, p-STAT3, PIAS3 and ALR expression in neoplastic human tissues from CRC patients, grouping the data in accordance with the histological alterations (G1, G2 and G3) and metastasis presence. Western blot (WB) analysis of ALR was also determined in neoplastic and surrounding tissues. Finally, cell proliferation (Ki-67) and apoptosis (Bcl-2) were determined.

RESULTS: Colon cancer tissue samples showed: (1) ALR and p-STAT3 strongly over-expression in 100% of G1 tissue samples, reducing in G2 and G3 tissue samples; (2) PIAS3 immunological determination was poorly expressed in G1 tissue samples and highly expressed in the 100% of colorectal tissues from group G2 and G3. Ki-67 progressively increases with the importance of the anatomic-pathological alterations and Bcl-2 resulted higher in G3 tissue samples compared to G1 neoplastic tissues. WB data evidenced, in neoplastic tissues, compared to the tumour-surrounding tissues, ALR over-expressed in G1 neoplastic tissues and down-expressed in G3 neoplastic tissues.

CONCLUSIONS: Our data demonstrate a different dynamism of the investigated factors in relation to the severity of CRC histological findings. We hypothesize that the positive expression of ALR and p-STAT3 in the neoplastic tissue samples from CRC G1 group, associated to the absence of PIAS3, could be useful marker to identify an early stage of the disease. Based on these data and on our previous studies on gut microbiota in precancerous intestinal lesions, we are confident that, after microbial priming, a cascade of molecular events is started. So, the detectable molecules acting in these initial steps should be considered for the study of CRC progression and therapy.

Key Words:
Colorectal carcinoma (CRC), Gut microbiota, Bacteroides, Clinical biochemistry, Signal Transducer and Activator Factors 3 (STAT3), Protein Inhibitor of Activated STATs (PIAS3), Augmenter of Liver Regeneration (ALR), Neoplastic progression, Laboratory medicine, Translational research.

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Introduction

Gastrointestinal cancers are very common and account for many deaths every year. Colorectal cancer, also called colon or large bowel cancer, includes cancerous growths in the colon-rectum and it is the third most common form of cancer and the second leading cause of cancer-related death in the Western World[1], preceding gastric cancers[2]. During the last years, a lot of scientific papers have linked intestinal dysbiosis to the origin of many cancers, especially the gastrointestinal ones. In fact, apart from the well-known role of *Helicobacter pylori* in gastric tumours[3-6], a certain modification of gut microbiota composition seems to be an effective promoter of intestinal oncogenesis, as recently reported by our group[7-12].

Many colorectal cancers are thought to arise from adenomatous polyps in the colon. These growths are usually benign, but they may develop into cancer over time. Although CRC shows a good prognosis when diagnosed at an early stage, it is frequently (more than 60%) diagnosed when it is diffuse and with the presence of metastases. CRC can take many years to develop and its early detection greatly improves the chances of a cure.

An important role, in the detection and treatment of patients with colorectal cancer, is covered by the possibility to evidence specific biomarkers, extremely necessary for tests able to identify, as early as possible, the development of a neoplastic process; the biomarkers, whose number continues to grow, have also the potential to change diagnostic and treatment algorithms by selecting the proper chemotherapeutic drugs across a broad spectrum of patients. The term of biomarkers, better tumor markers, refers to substances (most typically proteins, glycolipids) representing biological structures, which can be attributed to the development of normal cells or carcinogenesis at different neoplastic development phases.

In the last decades, many aspects of the molecular and biological pathways of CRC have been understood. It is known that the interaction between genetic and environmental patterns requires long periods to determine the progression of CRC. It is now possible to recognise preneoplastic and early neoplastic lesions, so that screening programs, based on colonoscopy and faecal occult blood test, are rapidly spreading in developed countries[13-16].

However, the exact intracellular pathways that determine the progression and confer invasivity to the tumour are not well characterized. It is evident that a disregulation of the balance between apoptosis and cell proliferation, towards apoptosis, could represent a good substrate for the carcinogenetic process as demonstrated by the up-regulation of Bcl-2 protein and TGFβ[17-20]. In particular, the apoptotic process could suffer inhibition, decreasing in expression and producing the activation of some related factors which lead to debilitation or inhibition of the process.
Among the factors which regulate this process, Signal Transducer and Activator Factors 3 (STAT3), Protein Inhibitor of Activated STATs (PIAS3) and Augmenter of Liver Regeneration (ALR) certainly hold important roles[21-23].

STATs belong to a family of cytoplasmic transcription factors, key mediators of cytokine and growth factors signalling pathways[24]. Seven STAT mammalian proteins have been identified so far[25,26]. Among those factors, STAT1, STAT3 and STAT5 have received great attention. Usually the binding of a cytokine to its cognate receptor on cell membrane induces a rapid and transient phosphorylation of STAT, its dimerization and translocation into the nucleus, where it activates target genes that control cell proliferation, survival, differentiation and development[27,28]. Recently, STAT3 and STAT5 have been implicated in the regulation of cell growth and malignant transformation, promoting chronic inflammation and increasing susceptibility of healthy cells to carcinogenesis[29], attributing to these factors a role of onco-proteins in the development of many tumours[30].

Moreover, a family of STAT proteins controlling factors has been identified, the Protein Inhibitor of Activated STATs (PIAS)[31]. Many components of this protein family have been recognized (PIAS1, PIAS3, PIAS5, PIASy), all of which regulate the transcriptional activity of many gene-regulatory factors through distinct mechanisms. PIAS3, for example, binds dimerized-STAT3 and inhibits its DNA-binding capacity, thus interfering with STAT3-mediated gene activation[31,32].

In 1994, our group reported the purification and the genetic sequence of a new protein from rat proliferating liver, called Augmenter of Liver Regeneration (ALR)[33,34]. ALR, a sulfhydryl oxidase enzyme[35,36] encoded by the growth factor erv1-like (Gfer) gene[37], is present in all mammalian tissues[34,37,38] and, compared to tissues from healthy subjects, is significantly up-regulated in tissues of patients affected by cell proliferating diseases, such as hepatocellular carcinoma (HCC), chronic hepatitis (CH), cholangiocellular carcinoma (CC)[39-41] or in regenerating liver after par-

tial hepatectomy in rats[42-44]. We have previously demonstrated that ALR protects human-derived neuroblastoma cells from H_2O_2-induced apoptosis, reducing mitochondrial swelling, cell death and apoptotic cell number, inhibiting cytochrome c mitochondrial release and up-regulating the Bcl-2/Bax gene transcription protein ratio[45]. Similar data are present, in literature, in different experimental models[46-48]. In a study, conducted in 70% partial hepatectomized rats, we detected the anti-apoptotic behaviour of ALR and, for the first time, we demonstrated its anti-oxidative capacity[45]. This latter physiological effect is achieved by the induction of the cytoplasmic/secretory form of clusterin (c-Clu)[45], one of the few chaperons secreted in the blood[49], highly expressed in aggressive late stage tumours[50] and, differently to its nuclear form (n-Clu), able to exert anti-apoptotic functions[50-53]. In addition, Dayoub et al[39] demonstrated that ALR expression in HCC cells attenuates cell motility, supports and maintains epithelial cell growth and it is inversely correlated with tumor grading and tumor angioinvasion, providing evidence that ALR may be considered as an antimetastatic protein in HCC, having the potential to become a marker in HCC diagnosis and a therapeutic target[39].

Following our translational research, aimed to investigate the clinical biochemical and environmental aspects which could determine and favorite the carcinogenetic process in the gastrointestinal tract, like reported in our most recent study on the role of gut microbiota in precancerous intestinal lesions[9,10], the present manuscript was realised to analyse the expression of three factors (ALR, p-STAT3 and PIAS3), controlling cell apoptosis and mixed up in the carcinogenetic process, in human colorectal cancer tissue samples, and were possible, in tumour-surrounding tissues, correlating these data with the histopathological findings, the presence of distant metastases, the expression of cell proliferation (Ki-67) and apoptosis (Bcl-2).

Patients and Methods

Clinical Cases

Human colorectal adeno-carcinoma tissue samples from 80 patients were provided by the Surgery ward of the Regional Hospital "X. Kongoli". The basic characteristic and clinico-pathological parameters of the 80 patients studied are reported in Table I. The group of 80 patients was composed of 44 males (median age 66 years, range from 36 to 82 years) and 36 females (median age 57 years, range from 28 to 81 years). Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, revised in 2013 as reflected in a priori approval by the Ethical Committee of the University of Elbasan (Research protocol Id: INTL_ALITMKCOOP/Cancerology/TRGTMED2019_KPBMT).

Histological Diagnosis

The histological diagnosis was established by two independent pathologists. Each tumour was assigned a histological type according to the World Health Organization (WHO) classification: well differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and mucinous carcinoma[54]. Neoplastic tissue samples are graded predominantly based on the extent of glandular appearances and divided in three groups: G1, G2 and G3. Well differentiated (G1) lesions exhibit glandular structures in >95% of the tumor; moderately differentiated (G2) adenocarcinoma has 50-95% glands, poorly differentiated (G3) adenocarcinoma has 5-50%.

Mucinous adenocarcinoma and signet-ring cell are considered poorly differentiated. According to the TNM staging system of the American Joint Committee on Cancer, the depth grading of tumour invasion in each of the carcinomas was classified into 4 groups: T1, invading submucosa; T2, invading muscularis propria; T3, invading either subserosa or pericolic tissue; T4, invading through serosa or invading contiguous organs[55].

Molecular Determinations

Tissue expression of ALR, PIAS3, p-STAT3, Bcl-2 and Ki-67 was determined, in all neoplastic tissues, by immunohistochemistry techniques. In addition, ALR expression was determined, by Western Blot analysis, both in neoplastic and in tumour-surrounding tissues of G1 and G3 groups, according to their availability.

Immunohistochemistry Techniques

ALR, p-STAT3 and PIAS3

Neoplastic tissue samples were fixed in 4% formalin, dehydrated in ethanol, clarified in xylene, and embedded in paraffin. Four μm thick samples were cut. Slides were then deparaffinised in xylene for 1 hour and progressively rehydrated

Table I. Demographic and clinic-pathologic parameters of the 80 patients enrolled. Tumour features are also reported.

Clinical parameters	Characteristic	No (%)
Gender	Male	44 (55%)
	Female	36 (45%)
Age-M		
Age-F	Range: 36-82 ys	
Range: 28-81 ys	Mean age: 66 ys	
Mean age: 57 ys		
Depth of invasion	T1	0 (0%)
	T2	12 (2.6%)
	T3	62 (77,5%)
	T4	6 (7.5%)
Lymph node involvement	N0	36 (45%)
	N1	44 (65%)
Distant metastasis	M0	64 (80%)
	M1	16 (20%)
TNM stage	I	18 (22%)
	II	22 (28%)
	III	20 (25%)
	IV	20 (25%)
Histologic grade	Well differentiated (G1)	12 (15%)
	Moderately differentiated (G2)	32 (40%)
	Poorly differentiated (G3)	36 (45%)
Tumor size	≥ 5 cm	48 (60%)
	< 5 cm	32 (40%)
Primary site	Right colon	22 (27.5%)
	Left colon	24 (30%)
	Rectum	34 (42.5%)

The basic characteristic and clinicopathologic parameters of the 80 CRC patients enrolled in the study and disease staging according to TNM classification: T1, tumor invades the submucosa; T2, tumor invades the *muscularis propria*; T3, tumor invades through the *muscularis propria* into the subserosa or perirectal tissues; T4, tumor directly invades other organs or structures and/or perforates the visceral peritoneum; N0, no regional lymph node metastasis; N1, lymph node metastasis; M0, no distant metastasis; M1, distant metastasis present.

through a graded descending ethanol series and washed in Phosphate-Buffered Saline (PBS) (Sigma-Aldrich, Milan, Italy). Antigen retrieval was performed dipping the sections in Citrate Buffer pH 6.0 and heating 3 times for 3 minutes each in a microwave oven at 700W. After washing in PBS, the sections were permeabilized by 0,25% Triton X-100 (Sigma-Aldrich, Milan, Italy) for 10 minutes (ALR) or 20 minutes (PIAS3, p-STAT3). After 3 washes in PBS, endogenous peroxidase was blocked in H_2O_2 for 10 minutes. Nonspecific protein binding was blocked by treating the sections with 5% Goat Serum (Sigma-Aldrich, Milan, Italy) dissolved in PBS for 30 min. The sections were then incubated overnight at 4°C with the appropriate antibody as follows: rabbit polyclonal anti-ALR (Multibind GmbH, Köln, Germany) diluted 1:200, rabbit monoclonal anti-phospho-STAT3 (Tyr 705) (Cell Signaling Technology, Cellbio, Milan, Italy) diluted 1:50, mouse monoclonal anti-PIAS3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200. Primary antibody preabsorbed with excessive recombinant p-STAT3 peptide (Santa Cruz Biotechnology, Inc, USA) was used as negative control. Prostatic tissue served as the internal positive control for p-STAT3 immunostaining[56]. After 3 washes with PBS, the sections were treated with the appropriate anti-rabbit or anti-mouse secondary EnVision antibody (Dako Italia, Milan, Italy) for 1 hour. Peroxidase labelling was visualized with 3-amino-9-ethylcarbazole (AEC, Vector Labs, Burlingame, CA, USA) as the chromogen. The slides were counterstained with hematoxylin and mounted and observed by optical microscope.

Ki-67 and Bcl-2 Immunohistochemistry Identification

Immunohistochemistry identification was performed using the automatic Dako Autostainer (Dako, Milan, Italy) and revealed by the LSAB method. Ki-67 sections were pre-treated for 30 minutes in pH 6.0 citrate buffer at 98°C. The primary antibody anti-Ki67 clone MIB-1 (Dako, Milan, Italy) was diluted 1:25. Bcl-2 sections were pre-treated for 30 minutes in pH 8.0 EDTA at 98°C. The primary antibody anti-Bcl-2 clone 100 (BioGenex, Medical Systems S.p.A., Genoa, Italy) was diluted 1:500. According to Kusaba et al[55], tissue samples were divided in two groups, depending on the percentage of cell specifically immune-stained. Tissue samples were considered negative when they showed specific immunoreactivity in less than 15 % of the observed tumour cells, while were considered positive when they showed specific immunoreactivity in more than 15% of tumour cells observed[55].

Western Blot Analysis for ALR Determination

Neoplastic and "normal" colonic tumour-surrounding tissue samples were suspended in ice cold radioimmunoprecipitation assay (RIPA) buffer (Pierce-RIPA buffer, Thermo Fisher Scientific, Rockford, IL, USA) with anti-proteases (Complete Mini protease inhibitor; Roche, Milan, Italy) and anti-phosphatases (sodium orthovanadate, 2 mM; Sigma Aldrich, Milan, Italy) freshly added, homogenized and centrifuged at 14,000 rpm for 15' at 4°C. Aliquots of 50 micrograms of protein concentration, determined by the standard Bradford assay (Bio-Rad Laboratories, Milan, Italy), were separated in a 4-12% pre-cast polyacrylamide gels (Invitrogen, Life Technologies, Monza, Italy) and transferred onto a polyvinylidene difluoride (PVDF) 0.2 μm membrane (Bio-Rad Laboratories, Milan, Italy) with Transblot Turbo (Bio-Rad Laboratories, Milan, Italy) for 12'. A 5% non-fat dry milk in T-BST blocking solution was applied and the membranes incubated overnight at 4°C in the presence of anti-ALR primary antibody produced against the conserved carboxy-terminal domain of the full-length human protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:500 in blocking solution. Then, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy) diluted 1:10,000 in T-BST for 1 hour at room temperature, and the complex detected by enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Rockford, IL, USA). To detect the β-actin housekeeping protein, the membrane was washed in the Restore Western Blot Stripping Buffer (Pierce Biotechnology, Illinois, IL, USA) for 15 min and reprobed with the anti-β-actin antibody diluted 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The densitometric analysis of each protein-related signal was performed using the Molecular Imager Chemidoc™ (Bio-Rad Laboratories) and normalized against β-actin related densitometric signal.

Statistical Analysis

The results obtained are expressed as mean ± standard deviation (M ± SD.). Statistical comparison among groups was determined using the Student's *t*-test. Statistical significance was ascribed to the data when $p < 0.05$. If not specifically reported, each datum is representative of at least three different and separated experiments. The analyses were performed using SPSS software, version 23.0 (IBM, Armonk, NY, USA).

Results

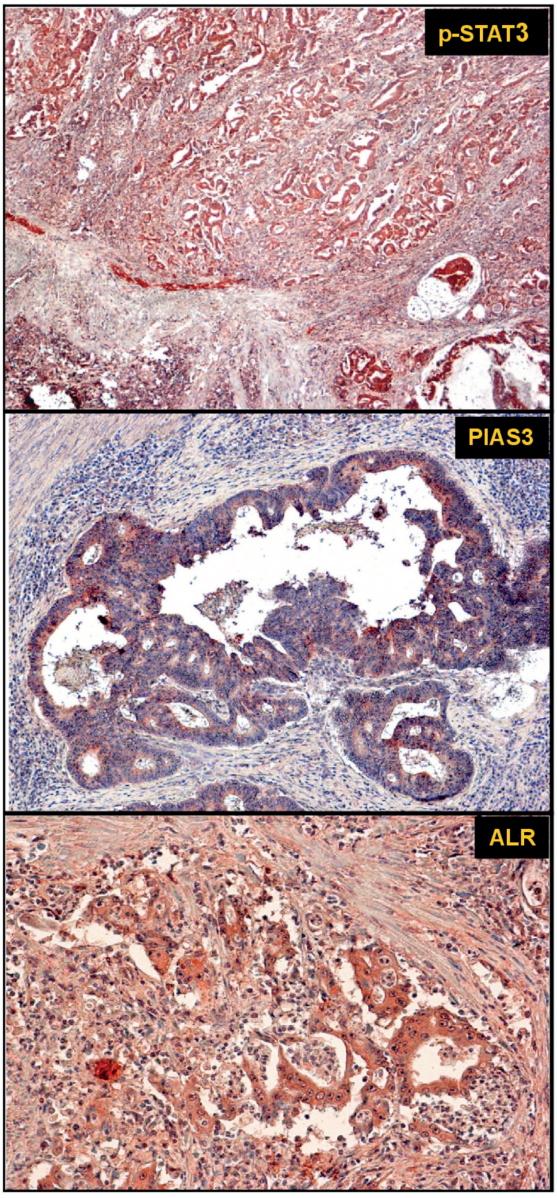
In the Table I, the demographic and clinic-pathologic parameters, together with the tumour size, metastases presence and organ localization of the 80 CRC patients are reported.

In Figure 1 the immunohistochemical identifications of p-STAT3, PIAS3 and ALR, determined on neoplastic tissue samples, are reported. In all three determinations a clear immune specific reaction was detected. Respectively, p-STAT3 immune-identification is shown in a tissue of an intestinal adenocarcinoma G1: an intense and diffuse cytoplasmic positivity for this factor is evident in the neoplastic glands. The immune identification of PIAS3 is reported in a tissue sample of an intestinal adenocarcinoma G2: an intense and diffuse cytoplasmic positivity for PIAS3 is evident in neoplastic cribriform glands surrounded by an inflammatory stroma. ALR immune detection is reported in a tissue sample of large bowel adenocarcinoma of histological grade G1: an intense cytoplasmic positivity for ALR in well differentiated neoplastic glands with necrosis and inflammation is evident. As reported in the Materials and Methods, the percentage of the neoplastic cells, independently positive for each of the factors studied, was considered.

Figure 2 reports the percentage of neoplastic tissue samples positive for the immune histo-

chemical expression of the three factors, grouped in accordance with their histological alterations (G1, G2 and G3). The neoplastic intestinal tissues from patients of group G1 where all ALR^+, gradually, but significantly ($p = 2.51 \times 10^{-9}$) reduced in the tissues of groups G2 (42.0 $\pm$ 4.3%) and G3 (33.6 $\pm$ 4.7%). p-STAT3 profile parallels the trend of ALR, being present in the 48.0 $\pm$ 4.4 % of neoplastic tissue samples of group G1, significantly reduced in the G2 group (30.2 $\pm$ 4.7%; $p = 2.19 \times 10^{-4}$) and reaching the lowest expression in the group G3 (18.8 $\pm$ 2.7%; $p = 1.79 \times 10^{-3}$). An opposite behaviour was observed for PIAS3 immuno-identification, positive in the 50 $\pm$ 6.8 % of neoplastic tissue samples of G1 group, significantly ($p = 2.18 \times 10^{-7}$) increased to the 99.4 $\pm$ 0.9 % of the neoplastic tissue samples of the G2 group and to the 100 % of the tissue from G3 group.

Figure 3 reports the percentage of neoplastic tissue samples, positive for the immunohistochemical expression of the three factors, separately considered in accordance with the absence (M0) or the presence (M1) of distant metastases. ALR was immunologically detected in the 61.4 $\pm$ 3.7% of tissue samples from M0-neoplastic tissues, significantly higher ($p = 1.0 \times 10^{-5}$) compared to the percentage (41 $\pm$ 2.9%) of M1-neoplastic tissues. Similar aspect demonstrated the



Intestinal adenocarcinoma G1: an intense and diffuse cytoplasmic positivity for p-STAT3 is present in neoplastic glands

Intestinal adenocarcinoma G2: intense and diffuse cytoplasmic positivity for PIAS3 is evident in neoplastic cribriphorm glands immersed in a inflammatory stroma

Intestinal adenocarcinoma G1: intense cytoplasmic positivity for ALR in poorly differentiated neoplastic glands with necrosis and inflammation was detected

Figure 1. Immunohistochemical identification of PIAS3, p-STAT3 and ALR in CRC intestinal tissues. As reported in Materials and Methods the immune-histochemical detection of p-STAT3 (40X), PIAS3 (100X) and ALR (100X) was separately determined in all the 80 CRC tissue samples. An example of the data obtained for each parameter is reported.

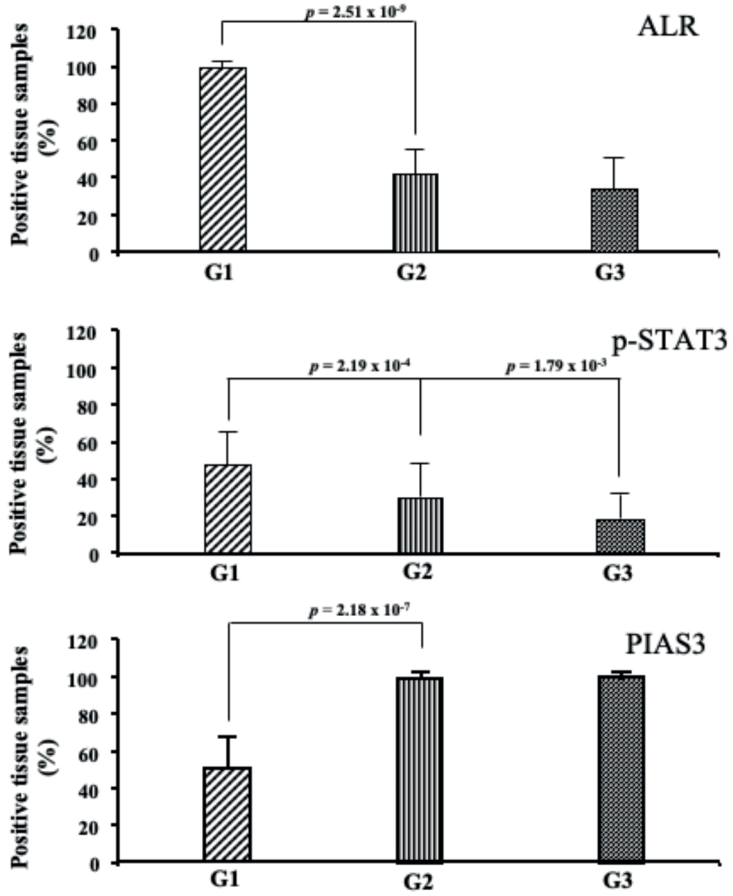


Figure 2. PIAS3, p-STAT3 and ALR immunohistochemical identification and CRC tumour grade. The percentage of CRC intestinal tissues, positive for each parameter (if it showed specific immunoreactivity in more than 15% of tumour cells), grouped according to the progressive severity of their anatomic-pathological findings, is reported.

immunodetection of p-STAT3, being positive in the 60.8 $\pm$ 3.9% of MO-neoplastic tissues and significantly (p = 5.1 x 10^{-5}) lower (42.8 $\pm$ 3.4) in the M1-tissue samples. Different behaviour was evidenced for PIAS3, significantly (p = 7.4 x 10^{-7}) lower in the MO-neoplastic tissues (61.2 $\pm$ 5.3% of samples) compared to the percentage (97.4 $\pm$ 2.6%) of the M1-neoplastic tissues.

Figure 4 reports cell proliferation (Ki-67) determined in tissue samples from CRC patients grouped in accordance to their histological grade (G1, G2 and G3). The 22.2 + 7.6% of tissue samples from patients of G1 group showed high expression of Ki-67, which increased in the G2 group of patients (29 + 20.1%), reaching the maximal and statistically significant expression (p = 4.3 x 10-3) in the neoplastic tissues of G3 group of CRC patients (50 + 14.5%).

Figure 5 reports the percentage of Bcl-2^+ cells in tissue samples from CRC patients grouped in accordance to their histological grade (G1 and G3). A statistically significant (p = 2.7 x 10^{-3}) greater percent of Bcl-2^+ cells was identified in the G3 group of CRC patients (65 $\pm$ 6.3%) compared to the neoplastic tissue from G1 CRC group of patients (30 $\pm$ 7.2%).

Figure 6 reports Western-blot ALR expression in tumour-surrounding and neoplastic tissue samples from G1 and G3 CRC patients. No difference was observed between the tumour-surrounding tissues from both groups (Figure 6A). Different was the behaviour of ALR expression in neoplastic tissue samples, compared to each respective tumour-surrounding tissue: a greater presence of ALR was registered in G1 neoplastic tissue samples, strongly reduced in G3 neoplastic tissue samples concerning, in particular, the cytoplasmic 21 Kd ALR isoform. The normalization of the densitometric protein-related signal against β-actin densitometric signal revealed a statistical-

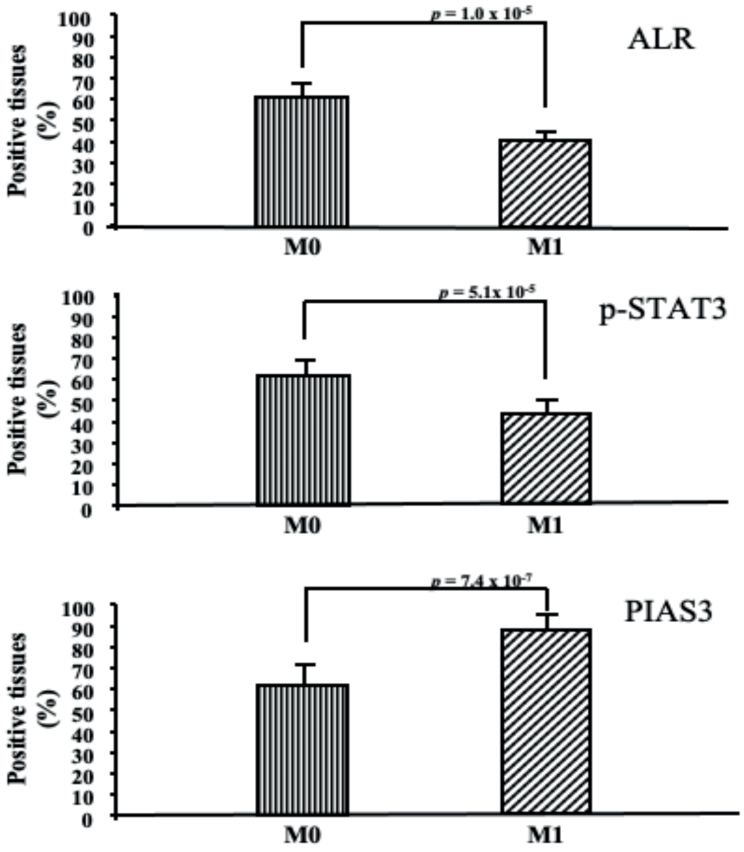


Figure 3. PIAS3, p-STAT3 and ALR immunohistochemical identification and distant metastasis presence. The percentage of CRC intestinal tissues positive for each parameter (if it showed specific immunoreactivity in more than 15% of tumour cells) and grouped according the presence (M0) or absence (M1) of distant metastases is reported.

ly significant difference between the tumour-surrounding and the neoplastic tissues in both the G1 and G3 tissue samples (p = 4.3 x 10^{-3} and p = 5.2 x 10^{-8} respectively). Similarly, the analysis of the data of the two neoplastic tissues densitometric protein-related signals revealed a statistically difference between the two groups (p = 3.7 x 10^{-9}) (Figure 6B).

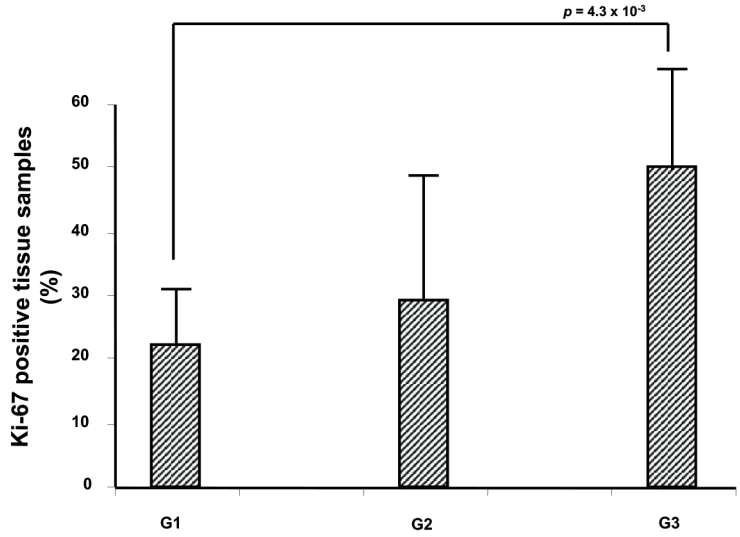


Figure 4. Proliferative activity of CRC tissues. Ki-67 levels determined in CRC intestinal tissue samples are grouped and reported according to the progressive severity of their anatomic-pathological findings.

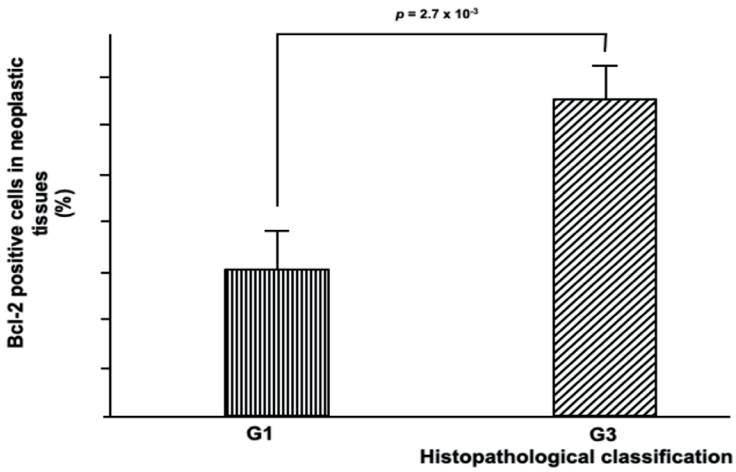


Figure 5. Anti-apoptotic protein expression. The percentage of Bcl-2 positive CRC intestinal tissue samples, grouped in accordance to their histological grade, is reported.

Discussion

In the present paper we report, in intestinal tissue samples from CRC patients, the behavior of three factors whose role in the regulation of the physiological mechanism of apoptosis is well documented: STAT3, an anti-apoptotic factor[21,56-59], PIAS3, a pro-apoptotic factor[60] and ALR, a factor with anti-apoptotic, anti-oxidative and anti-metastatic activities[40-47].

The analysis of cell proliferation (Ki-67; Figure 4) and apoptosis (Bcl-2; Figure 5) in the neoplastic tissues seems to confirm the data reported in literature. The data of Bcl-2, maximally-expressed in tissue samples from G3 group, could appear in contrast with the anti-apoptotic activities of ALR and p-STAT3, strongly immunologically expressed in tissue samples of G1 group (Figure 2) but, actually, they confirm that in the neoplastic cells does not exist a direct relationship between the apoptosis regulatory factors and the anti-apoptotic gene expression as it occurs in normal tissues[61].

Of particular interest is the analysis, as a whole, of ALR, p-STAT3 and PIAS3, that highlights two aspects: (i) ALR and p-STAT3 are, immunohistochemically over-expressed in CRC from G1 group, compared to similar data from neoplastic tissues of G2 and G3 CRC groups; (ii) PIAS3, slightly expressed in G1 tissues, is strongly expressed in G2 and G3 group tissues (Figure 2). Moreover, grouping the immunological results of neoplastic tissues and comparing with the presence of distant metastasis, ALR and p-STAT3 positive tissues are statistically higher in neoplastic tissues from patients without metastases (M0) compared to neoplastic tissues from patients showing metastases (M1) (Figure 3). On the contrary, a higher percentage of neoplastic tissues, immunologically positive for PIAS3, was evidenced in CRC patients showing metastases (M1) compared to CRC patients without metastasis (M0) (Figure 3).

According to these data, we believe that it could be possible to identify two different phases of the neoplastic process proceeding from well differentiated (G1) lesions to moderately differentiated (G2) adenocarcinoma and finally to poorly differentiated (G3) adenocarcinoma: a first phase, characterized by G1 data, including neoplastic tissue samples strongly positive for ALR and p-STAT3 and slightly positive for PIAS3, and a second phase, characterized by G2-G3 data reporting results from neoplastic tissues strongly positive for PIAS3 and weakly positive or negative for ALR and p-STAT3 (Figure 3).

Our considerations on immunohistochemistry data are supported by WB analysis of ALR in both neoplastic and tumour-surrounding tissues, for which study we, specifically, targeted the two anatomopathological extreme conditions of CRC subjects studied, G1 and G3 (Figure 6). The data obtained showed two, apparently different, panels of ALR expression in neoplastic tissues compared to tumour-surrounding tissues: (i) a statistically ($p = 4.3 \times 10^{-3}$) greater expression of ALR in G1 tissues, and (ii) a statistically ($p = 5.2 \times 10^{-8}$) lower ALR expression in G3 tissues. In tumour-surrounding tissues of both CRC histopathological classifications ALR expression resulted superimposable.

The neoplastic tissue data may appear to conflict with each other even if, both, are in accordance with the data of the literature: indeed, on one hand G1 results confirm similar data reported in all neoplastic tissues analysed, which report an

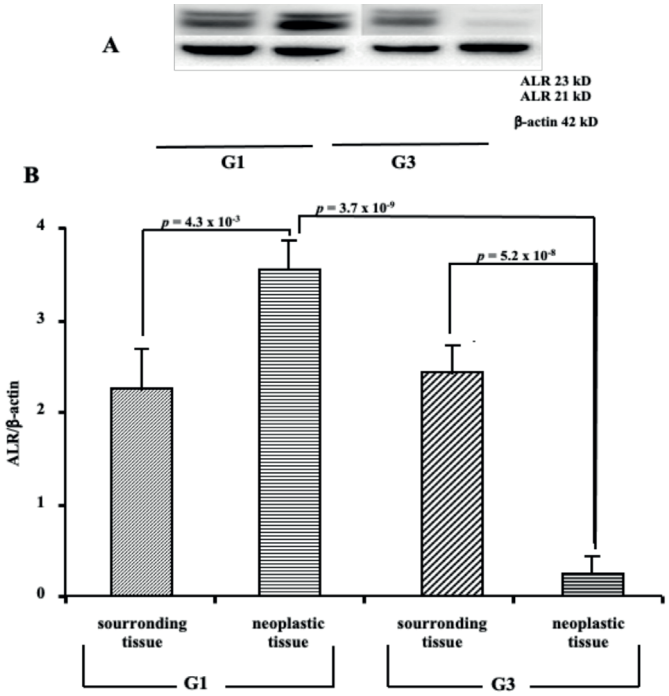


Figure 6. ALR expression, by Western blot analysis, in tumour-surrounding and neoplastic tissues samples from G1 and G3 CRC patients (A). Protein densitometric normalization, by β-actin immunodetection, is reported in the B section.

ALR over-expression[39-41], on the other hand, G3 results are supported by previous data, reporting an inverse correlation between ALR expression and tumour grading and angioinvasion[40,41].

We believe that they represent the dynamism of the molecular alterations that characterize the biological evolution of the carcinogenetic process, as far as we know, not evidenced so far. Indeed, if the G1 results, ALR over-expression, could represent a physiological attempt of an anti-metastatic factor against the neoplastic cell proliferation[40,41], the G3 results may represent a biological situation probably due to a different, uncontrolled, gene expression, typical of neoplastic-undifferentiated cells. In addition, the increase of an anti-apoptotic, anti-oxidative and anti-metastatic factor, in the initial step of the carcinogenetic process (G1) when the tumour tissue looks still well-differentiated, could be necessary to make the epithelial cells resistant to the metabolic changes induced by the neoplastic molecular variations and protect the body from an early metastatic spread.

In summary, the ALR conflicting results may shed some light in the molecular evolution of the neoplastic microenvironment, at least for CRC, supporting the evidence for potential involvement of ALR and of p-STAT3, at the early stages of the malignant progression, which, we believe, strongly progresses when these factors are down regulated.

Many reports are present in the literature concerning the role and the involvement of p-STAT3 and PIAS3 in the neoplastic process[56-60]. The linkage between STAT3 activation (p-STAT3) and colon carcinogenesis has been frequently reported in the last twenty years. First, Ma et al[61] concluded, studying STAT3 expression in tissue samples from patients of primary colorectal carcinoma, that the constitutive activation of STAT3 may play an important role in the tumorigenesis of colorectal carcinoma[61].

More specifically, recently, Zizi-Sermpetzoglo et al[62] reported, in CRC tissue samples, a statistically significant correlation between p-STAT3 immunoreactivity and poor CRC differentiation having observed, by immunohistochemistry, a significantly greater presence of p-STAT3 expression in well-differentiated neoplastic tissues compared to poorly differentiated CRC[62]. The authors conclude that p-STAT3 is an important factor related to CRC carcinogenesis and is significantly associated with some clinicopathological parameters, such as depth of tumour invasion, advanced Dukes stages, and lymph node metastasis[63].

Studying, in more depth, the apoptotic process and the relationships between the factors that control it (p-STAT3 and PIAS3) during the progress of carcinogenesis, like herein we reported, Saini et al[63], observing the development of serous carcinoma from the fallopian tube (FT), reported that in-

creased expression of activated STAT3 (p-STAT3 Tyr705) and suppression or loss of PIAS3 in FT likely drive high-grade serous ovarian carcinoma (HGSC), concluding that the activation of STAT3, under the influence of decreased levels of PIAS3, is an early event in the initiation of malignant transformation, and that these changes govern the neoplastic transformation to HGSC[63].

Conversely to STAT3 and PIAS3, few and not clear are the data about the role of ALR in a carcinogenetic process. It has previously been demonstrated, in different pathophysiologic situations, that the main role of ALR over-expression is to contrast both the cellular oxidative process and the apoptosis as well the metastatic spreading[40,41,43-47]. It is well known that apoptosis is a molecular process that takes place in three different biological situations: 1. the apoptosis that occurs under physiological conditions and that, as in its original etymological meaning, it stands for "felling of", "fade naturally, gradually", ensuring the renewal of the tissues; 2. the apoptosis, that we call "regenerative apoptosis", that occurs in pathological reversible conditions such as in the experimental model of liver regeneration after partial hepatectomy in rats. In this latter experimental condition, we have shown that a crucial role is covered by ALR, which determines a considerable reduction of "cell death" preserving a greater number of cells for the regenerative process; similarly, the apoptosis that occurs in the neoplastic tissue, that we identified as "onco-protective apoptosis", could be a process in which ALR exerts its anti-apoptotic capacity[65] together with its anti-metastatic activity[40].

The most important suggestion of the present research comes from the demonstration of the different tissue expression of the pro- and anti-apoptotic factors (ALR, p-STAT3 and PIAS3) in relation to the severity of the disease (Figures 2 and 3). It is obvious that the identification of two groups of patients, one in which ALR and p-STAT3 result immuno-histologically positive and PIAS3 negative, and another one in which ALR and p-STAT3 are negative and PIAS3 strongly positive, represent two extreme biological situations. At the beginning of the carcinogenetic process, the biochemical behaviour of the neoplastic tissue is finalized to the maintenance of a sustainable grading of disease, conversely, progressing the neoplastic process, the molecular changes of tumour cells are so extensive and consolidated to compromise any physiological preservation of the tissue and its genetic expression.

As summarized in Figure 7, we must consider intestinal dysbiosis as the primer of tumorigene-

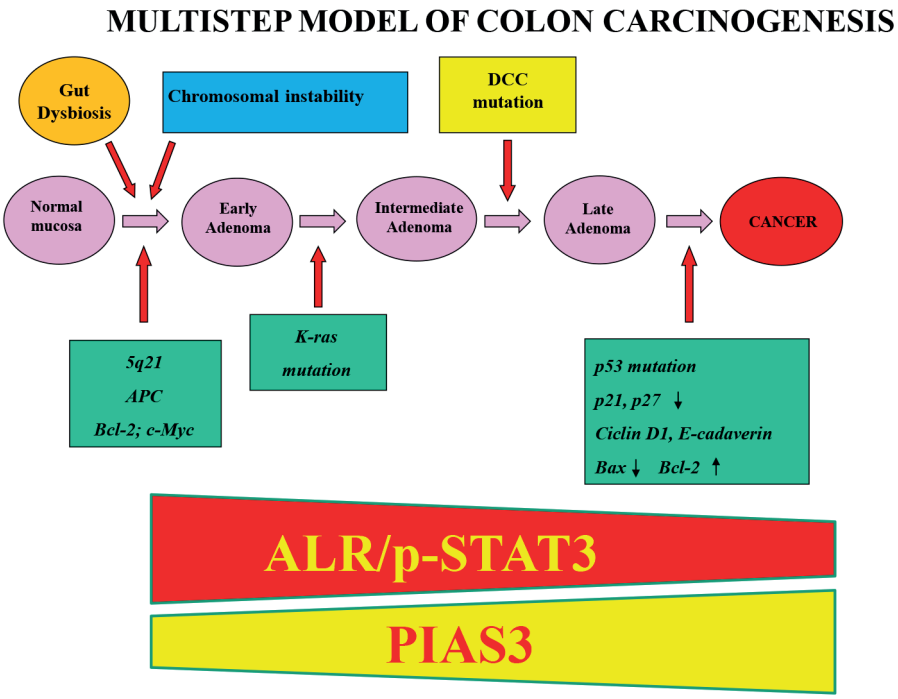


Figure 7. Multistep model of colon carcinogenesis including the immuno-detection of ALR, p-STAT3 and PIAS3 in the intestinal samples of CRC patients.

sis in CRC patients, an event followed by a cascade of cellular and tissue molecular changes[66-71], and we suggest that the analysis of gut microbiota composition should be added to the screening protocols for high risk people, as well as ALR, p-STAT3 and PIAS3 should be titrated in patients diagnosed for intestinal polyps and cancers.

Indeed, our previous data from patients with intestinal polyposis, showed a different composition of gut microbiota compared to healthy people, revealing the presence of bacteria with antiproliferative activity on colon cancer cells in fecal samples from people without intestinal polyps (e.g., *Pediococcus pentasaceus*) and the presence of bacteria species known to be associated to colon carcinogenesis (i.e., *Bacteroides fragilis* and *Prevotella melaninigenica*), was detected in people with intestinal polyps[9,10].

These findings are supported by previous studies reporting that similar bacterial species and, probably, many other are involved in the development of intestinal carcinogenesis, modulating the inflammatory, proliferative and apoptotic processes[7,8,71-73].

According to this idea, it will be possible to stop the initiation of CRC at its earliest stages modulating the composition of the microbiota including similar mucosal microbiota (i.e., oral and vaginal) and of the first oncogenic molecular steps[74-83] by the administration of probiotics and/or targeted drugs[84-94].

Conclusions

Based on our previous and current results we believe that the currently accepted scheme of CRC priming and evolution should be implemented by the analysis of ALR expression, correlating it with the anatomopathological grading for a more accurate determination and, possibly, an early diagnosis and consequent therapeutic choice. Starting from these considerations, the immunological expression of ALR and p-STAT3 in CRC tissue has to be considered important for the definition of the early phases of the disease (G1), while PIAS3 evaluation seems to be more determinant in the definition of the late phase of the neoplastic process (G2 and G3).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Author Contributions

L.P and A.F. conceived the study and were responsible for tissue culture and manuscript writing. D.P. and M.G.F. contributed to laboratory analyses. R.P. and A.D. helped to draft the manuscript and contributed to bibliographic research. A.B. and K.H. contributed to data analysis and interpretation, and manuscript revision. L.S. and S.K. made substantial contributions to experiments and coordination and supervised the manuscript for the final approval. All the authors read and approved the final manuscript.

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