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The Lauren Classification Highlights the Role of Epithelial-to-Mesenchymal Transition in Gastric Carcinogenesis: an Immunohistochemistry Study of the STAT3 and Adhesion Molecules Expression

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ABSTRACT

Background & Aims: Despite some recent advances, gastric cancer remains an important cause of death at world level. This indicates an absence of therapeutic options, stemming from the limited understanding of the molecular mechanisms involved in carcinogenesis. Nearly fifty years ago Lauren classified gastric cancers, according to the morphological aspect, as intestinal or diffuse. The phenotype of the cells indicates the presence of different molecular mechanisms, which can be approached in the light of recent data and identified with the help of current techniques. The best described are the germline/somatic mutations or the hypermethylations of the E-cadherin 1 *CDH1* gene promotor.

Methods. We analyzed 195 gastric tumors,120 intestinal and 75 diffuse type, using immunohistochemistry (tissue microarray TMA method) for pStat3^{Tyr705}, E-cadherin, α-catenin and β-catenin; 985 spots of gastric tumors, distributed on 4 TMA blocks were analyzed. For pStat3^{Tyr705} we took the nuclear staining into account and for the adhesion molecules, membrane staining.

Results. In our study, in the diffuse type gastric cancer, pStat3^{Tyr705} nuclear expression was statistically significantly increased ($p=0.003$). Also we observed a decreased expression of the adhesion molecules in the same type of gastric cancer (E-cadherin $p<0.0001$, α-catenin $p<0.0001$, β-catenin $p<0.0001$), suggesting that epithelial-to-mesenchymal transition (EMT) may be involved not only in gastric carcinogenesis, but also in resistance to treatment.

Conclusion. The Stat3 role has been recently highlighted in carcinogenesis of the diffuse type of gastric cancer. We found that the morphological features of the diffuse type also suggest the involvement of EMT in this type of gastric cancer. Therefore, targeting the key molecules involved in this process may interfere with EMT process in the diffuse type of gastric cancer.

Key words: gastric cancer – Stat3 – adhesion molecule – EMT.

Abbreviations: CDH1: E-cadherin 1; EMT: epithelial-to-mesenchymal transition; STAT 3: signal transducer and activator of transcription 3

INTRODUCTION

Gastric cancer is a major health problem worldwide; the mortality of patients with this type of tumor is second only to lung cancer [1]. In recent years, new targeted drugs were synthesized in order to inhibit molecules involved in gastric carcinogenesis, such as HER2 and c-Met. Despite these advances, survival rates at 5 years remain under 30%, proving a still limited understanding of the molecular mechanisms involved in gastric carcinogenesis [2]. According to the morphological features, Lauren's and also more recently the WHO classification distinguish two types of gastric adenocarcinoma, intestinal (with a papillary, tubular and mucinous architecture) and diffuse (poorly cohesive carcinoma), the latter being usually associated with lower survival rates [3]. These morphological differences indicate the presence of different molecular mechanisms (mutations of *p53, PTEN, PIK3CA, ARID1A* genes and amplifications of *HER2* oncogene), of which the best described are the germline/somatic mutations or the hypermethylations of the *CDH1* gene, encoding adhesion molecule E-cadherin [4-6]. Attempts have been made to identify new subgroups

on the basis of new molecular data, which partially overlap the classical histopathological classification based upon the morphological features of tumor cells [7]. Studies published over the last few years have described the involvement of the signal transducer and activator of transcription 3 (STAT3) in various types of tumors, including gastric cancer [8-12]. The activation of Stat3 involves a less favorable prognosis and a more limited response to treatment [16-18]. As a transcription factor, STAT3 acts by controlling the expression of some genes that regulate the cellular cycle or inhibit apoptosis, such as Bcl-2, Mcl-1, survivin, p53, c-Myc, cyclin D1, through a transduction pathway leading to an activated phosphorylated state which allow STAT3 the translocation into the nucleus [13-15]. A recent study indicates the involvement of Stat3 in the epithelial-to-mesenchymal transition (EMT) by way of a mechanism that leads to the nuclear localization of Snail, a transcriptional repressor for *E-cadherin 1* (*CDH1*). This causes a change in the cellular phenotype, as the cells become round, loose intercellular contact and acquire invasive capabilities [19]. The involvement of Stat3 in EMT, followed by an increased invasion and metastasis of tumor cells, has been reported in cases of breast and prostate cancer [20-22].

Starting from the particular morphology of the diffuse type, with poorly differentiated, small, round, dissociated cells, but also from the data in the literature which suggest that an EMT process is present in this type of gastric cancer, we sought to perform an immunohistochemical assessment of the expression of activated form of STAT3 ($pStat3^{Tyr705}$) and also of the adhesion molecules E-cadherin, α and β-catenin, in the two main types of gastric cancer defined by Lauren.

MATERIAL AND METHOD

Patients

Tumor fragments from 195 patients suffering from gastric cancer were retrieved from the archives of the pathology departments at the Gustave Roussy Institute of Oncology (Paris, France), Montpellier Cancer Institute (Montpelier, France), Ion Chiricuta Oncology Institute (Cluj Napoca, Romania) and La Sapienza University Hospital of Rome (Rome, Italy). Prior to the construction of the tissue microarray blocs, the cases were independently reviewed by two pathologists called upon to confirm the diagnosis of intestinal or diffuse gastric cancer according to the Lauren classification. None of the patients had received pre-operative chemotherapy. We examined the medical records of each patient in order to determine gender, age, tumor size, the number of metastasizing lymph nodes and the presence of metastases.

Construction of tissue microarray blocs

This was done with the help of a manual tissue microarrayer (Beecher Instruments®, MD, USA). Four cores (0.6 mm in diameter, 4 mm in length) were extracted for each patient's formalin fixed paraffin embedded block. The cores featured the optimum number of tumor cells [minimum 60%], as the areas rich in stroma or with necrosis had been avoided. Several cores coming from a healthy gastric mucosa were inserted into the tissue blocks, allowing us to assess the sensitivity and the specificity of the antibodies employed.

Immunohistochemistry

The immunohistochemical staining tissue sections (4 μm) were deparaffinized in xylene (3x15 minutes) and rehydrated in a graded series of ethanol (3x10 minutes). The activity of endogenous peroxidases was blocked through incubation with hydrogen peroxide [0.3%] in methanol at room temperature (2x15 minutes). After rinsing in tap water, the antigen retrieval was completed in microwaves (pH 7), 15 minutes at 750W and then 15 minutes at 150W. The immunohistochemical stain was performed using the following dilution for primary antibodies: 1:50 for $pStat3^{Tyr705}$ (Cell Signaling Technology® MA, USA), 1:50 for E-cadherin (BD Transduction Laboratories™ CA, USA), 1:50 α-catenin (Life Technologies™ NY, USA) and 1:50 β-catenin (Life Technologies™ NY, USA). The visualization of antibodies was made using the Envision DAKO® technique: after applying the primary antibodies at the indicated dilutions (1 hour at room temperature), we applied the secondary antibody Envision/HRP (45 minutes), followed by the application of Streptavidin in conjugation with peroxidase (45 minutes). The counterstaining of slides was done with Mayer's hematoxylin, and then they were rinsed, dehydrated in graded ethanol followed by xylol and mounted.

The immunohistochemistry interpretation

We assessed the percentage of tumor cells (0–100%). The immunomarking was classified according to location (membrane, nucleus, or cytoplasm) and intensity (absent, poor, moderate, intense). The slides were independently read by two pathologists. In the case of divergent results, the slides were reviewed by both pathologists working together, and an agreement was reached.

Statistical analysis

The statistical analysis was conducted using the Student *t* test. P-values of <0.05 were regarded as statistically significant. The data were analyzed using Prism 5.0b (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel 2008 (Version 12.1.9; Microsoft Corporation, Redmond, WA).

RESULTS

The studied population included 195 patients, of whom 75 suffered from gastric adenocarcinoma of the intestinal type and 120 of the diffuse type. Eighty four were women and 111 were men, the average age being 68 years (range 30 to 89 years). The size of the tumor, the number of metastasized lymph nodes and the presence or absence of distant metastases are shown in Table 1.

We analyzed 985 spots distributed on 4 TMA slides. For $pStat3^{Tyr705}$ we took into account only the nuclear marking (Fig.1), and for the adhesion molecules (E-cadherin, α-cadherin, β-cadherin) we took into account the membrane marking (Figs. 2-4). $pStat3^{Tyr705}$ was mainly expressed in diffuse type of gastric cancer (median 31.2, and the standard deviation 41.2), leading to a statistically significant value ($p=0.003$). For the adhesion molecules the expression was decreased in the same type of gastric tumor: E-cadherin – median 62.0, standard deviation 54.5 and $p<0.0001$; α-catenin – median 38.1, standard deviation 46.3 and $p<0.0001$; β-catenin – median 47.9, standard deviation 47.1 and $p<0.0001$ (Table II).

Table I. Pathological characteristics of the 195 studied patients with gastric cancer.

T	1	15 (7.72%)
	2	80 (40.9%)
	3	51 (26.4%)
	4	49 (25.4%)
N	0	41 (21.2%)
	1	73 (37.3%)
	2	45 (22.8%)
	3	33 (17.1%)
	x	3 (1.55%)
M	0	94 (48.2%)
	1	45 (23.3%)
	2	56 (28.5%)

T: tumor; N: node; M: metastasis

pStat3^{Tyr705} expression in intestinal and diffuse type of gastric adenocarcinoma. The expression is nuclear in tumor cells of diffuse type, weak citoplasmatic in normal glands (Fig. 1A) and tumor cells of intestinal type (Fig. 1B). E-caderin is diferently expressed in the two types of gastric cancer, as seen in Figs. 2 A,B. Alpha-catenin expression is increased in intestinal type compared with the diffuse type, as shown in Figs. 3 A,B. Beta-catenin has a weak expression in the diffuse type and a strong membranary positivity for intestinal type, according to Figs. 4 A,B.

Table II. Markers' expression in intestinal and diffuse type of gastric cancer.

	Diffuse type 75 patients		Intestinal type 120 patients		p value (T test)
	Median	Standard deviation	Median	Standard deviation	
pStat3^{Tyr705}	31.2	41.2	14.1	33.0	0.003
E-cadherin	62.0	45.4	99.2	9.1	< 0.0001
α-cadherin	38.1	46.3	98.3	12.9	< 0.0001
β-cadherin	47.9	47.1	95.5	19.1	< 0.0001

DISCUSSION

In our study, we highlighted the statistically significant difference of expression for pStat3^{Tyr705} and also for the adhesion molecules (E-cadherin, α and β- catenin) in the intestinal and diffuse type of gastric cancer. The genetic programs involved in the normal development are highly preserved, and their abnormal activation can be responsible for the occurrence and progression of malignancies [22-24]. Epithelial-to-mesenchymal transition is essential for embryonic development and tissue remodeling, but also for tumor invasion, metastasis and treatment resistance [25]. From a molecular point of view, the hallmark of EMT is the decreased E-caderin expression and the activation of transcription factors such as SNAIL, SLUG, TWIST1, and also STAT3 [26, 27]. The interaction between the aforementioned transcription factors can also be found within malignancies, as they are involved in the generation and the progression of the tumor [28-31]. These molecular

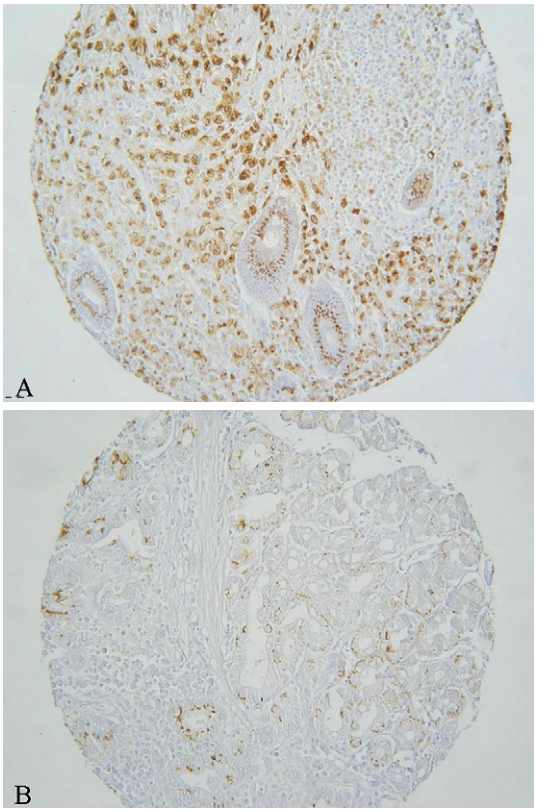

Fig. 1. pStat3^{Tyr705} expression in normal glands (A) and tumor cells of intestinal type (B) (x10).

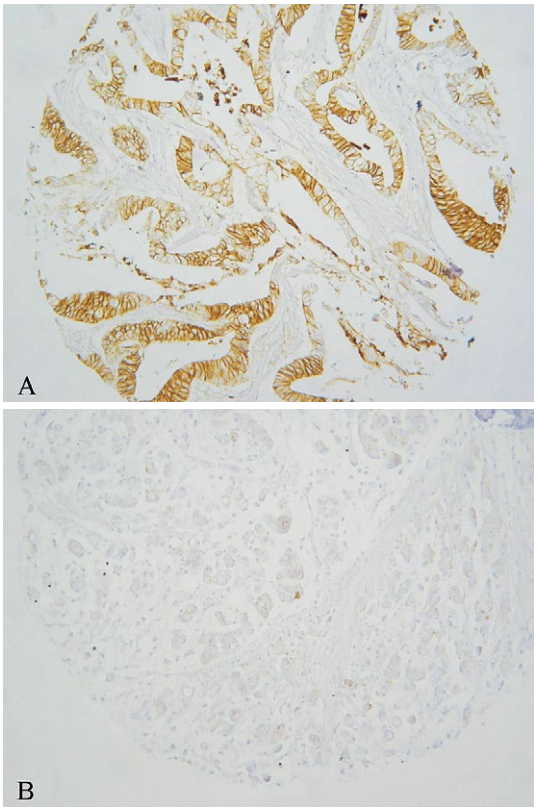

Fig. 2. E-Cadherin expression in the two types of gastric cancer (intestinal, A and diffuse, B) (x10).

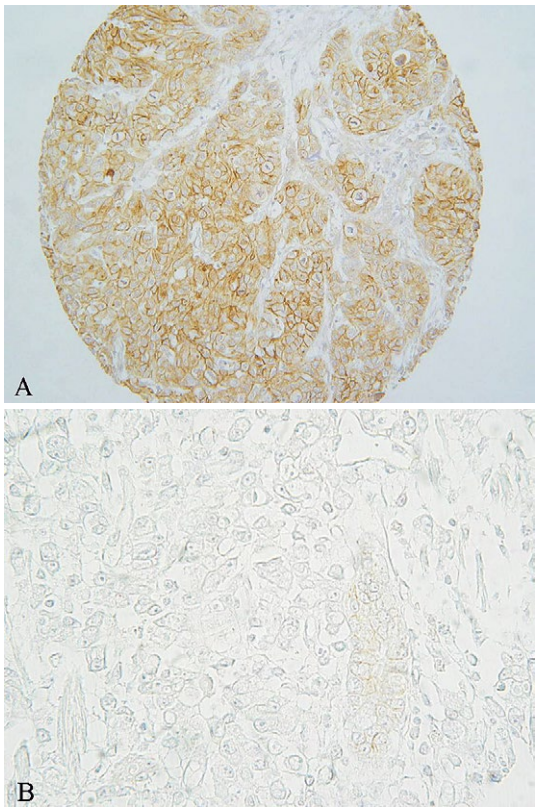

Fig. 3. Alpha-catenin expression in the intestinal type (A) and the diffuse type (B) (x10).

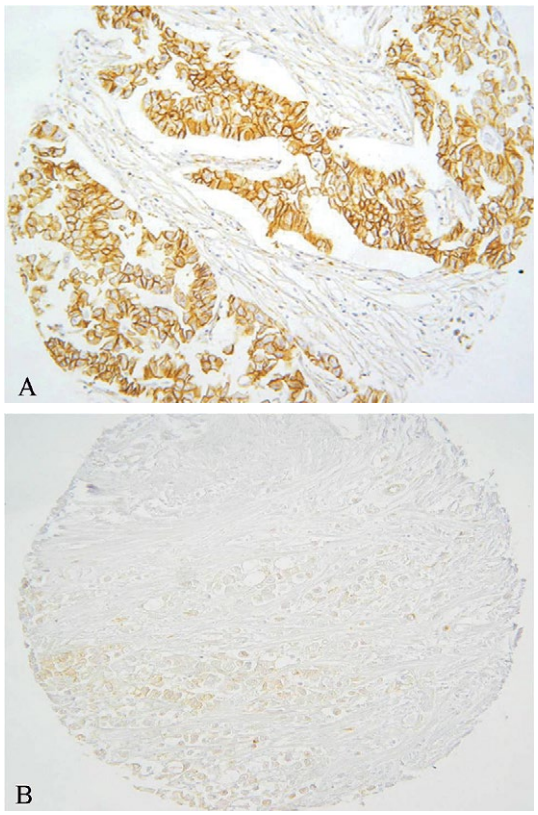

Fig. 4. Beta-catenin expression in the intestinal type (A) and the diffuse type (B) (x10).

events are reflected in the cell phenotype, the transition from an epithelial phenotype to a mesenchymal one leading to a decrease in the expression of adhesion molecules, to a loss of cellular adhesion, of polarity, followed by the detachment of cells that acquire invasive properties, just as in the case of diffuse gastric carcinoma [32].

The involvement of Stat3 in gastric carcinogenesis has been described, but the precise mechanism is yet to be determined [33-37]. In vitro studies have revealed the role played by Stat3 in cell proliferation, motility and invasiveness [38-40]. Similarly, in vivo animal studies have highlighted the fact that Stat3 has an important role to play in gastric tumorigenesis [41-43]. STAT3 activation is made by tyrosine phosphorylation at the single site (Y705) and is mediated by a Janus kinase. This is required for the Stat3 dimerization, nuclear translocation, and the DNA binding. The study of JAK and STAT knockout animals suggests that the JAK-STAT signaling pathway is important for development although not all the JAKs and STATs are equally essential. All the STAT genes and proteins have been located in bone tissues. Among the seven STATs, only STAT5A and STAT5B knockout mice show obviously defective development [30-32]. STAT5A and STAT5B are functionally quite pleiotropic. Biochemical and genetic studies have underscored the important role that STAT5A and STAT5B plays in directing a biological response to the IL-3 (IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor), single-chain (e.g., growth hormone, prolactin, thrombopoietin, and erythropoietin), and γc (i.e., the IL-2, IL-7, IL-9, IL-15, and possibly IL-21) receptor families. The extensive sequence similarity between STAT5A and STAT5B (~96% amino acid identity) explains their functional redundancy. But, the responses to prolactin and growth hormone favor STAT5A and STAT5B, respectively. STAT3 regulates cell respiration in mitochondria besides its action on gene transcription via binding specific gene-promoter sequences in nucleus. Without STAT3, for instance, an electron transport chain in mitochondria is inhibited leading to accumulation of reactive oxygen species (ROS) in adult cells. Clinical evidence suggests interplay between oxidative stress and carcinogenesis. One reason is that oxidative stress antagonizes wnt signaling. Wnt molecules, on the other hand, have an anabolic effect on tissue formation. Future research includes identification of downstream genes affected by JAK-STAT pathway in adult cells. The STAT3 signaling upregulates the expression of receptor activator of nuclear factor κB ligand (RANKL) in cells, suggesting that the IL-6-gp130-STAT3 signal regulates the differentiation indirectly. However, the role of STAT3 *in vivo* has not been elucidated. This can be achieved using a conditional knockout mouse model in which STAT3 inactivation occurs specifically in the malignant cell. IL-6, one of the main chemokines present in patients serum, promotes EMT by repressing E-cadherin expression via the JAK/STAT3/Snail signaling pathway.

Using immunohistochemistry, we highlighted a difference in the nuclear expression of $pStat3^{Tyr705}$ between the two types of gastric cancer in the Lauren classification, intestinal and diffuse. This difference in expression was correlated with that of the adhesion molecules E-cadherin, alpha and beta catenin, typical for the cells undergoing EMT. Few studies have analyzed the immunohistochemical expression of

pStat3^{Tyr705}, and it has been recently reported that a difference in the expression of pStat3^{Tyr705} correlates with the degree of tumor differentiation (I/II versus III/IV]), the TNM stage, and survival [44]. The same study indicates that STATs target the genes involved in the signaling pathways MAPK and mTOR, but also in Wnt, which is known to be involved in the preservation of an undifferentiated cell phenotype in normal and pathological conditions. On the other hand, in a recent study which correlates the immunohistochemical expression with the clinical-pathological parameters, Lee et al. have found a correlation between pStat3^{Tyr705} expression and survival, but this value remained statistically irrelevant when comparing the intestinal and the diffuse type [45]. The unphosphorilated Stat3 evaluation seems not to be prognostically relevant, as another study has failed to identify a statistically significant difference in survival for the positive and the negative STAT3 groups [46]. Another immunohistochemical study has found a significant correlation between early and advanced gastric cancers, but has not identified a difference between the intestinal and the diffuse types, the latter being positive for a small number of cases [47].

Although *in vitro* studies conducted on animal models indicate the important role of Stat3, the immunohistochemical evaluation and its correlation with clinical-pathological factors require considerable caution. The evaluation of the nuclear expression of the activated form (phosphorylated at Tyr705) is the only one that correlates between the degree of differentiation and the clinical evolution of patients. In our study, the comparison between the intestinal and the diffuse type has highlighted a difference for pStat3^{Tyr705}. The more significant expression of pStat3^{Tyr705} at the level of the cells molecularly characterized by a low expression of adhesion molecules suggests that Stat3 acts by way of a mechanism associated with the EMT process. This can account for the data obtained *in vitro* for Stat3 regarding the increase in survival, in resistance to treatment, proliferation and invasiveness, all associated with a loss of the epithelial phenotype, dedifferentiation, leading to the so-called stem-like status. This is corroborated by the involvement of Stat3 in EMT during embryogenesis, but also in tumor processes [23, 24].

Studies conducted in vitro on hepatocellular carcinoma cells, non-small cell lung cancer cells, cisplatin-resistant ovarian cancer cells, but also on squamous cell carcinoma of the esophagus, have indicated that blocking the activated Stat3 inhibits the EMT and might be a therapeutic alternative, leading to a better response to treatment and survival [48-51]. In the case of gastric cancer, the involvement of STAT3 in the resistance to treatment has been reported in the literature [52-56]. Furthermore, this resistance is associated with the activation of Stat3 and EMT [19, 57]. The molecular interaction of STAT3 and Snail, ZIP6, and also the other transcription factors suggests that it is part of a core that controls EMT in normal and pathological conditions [58, 59]. These data highlight the central role of STAT3, which could suggest that it could be a therapeutic target, its inhibition leading to a decrease in the main characteristics of tumor cells: proliferation, motility, invasion and upregulation of apoptosis. Today, there are a number of STAT3 inhibitors, such as the oligonucleotide decoy, antisense oligonucleotide or small molecules that are currently in clinical development [60, 61]. According to our study, pStat3^{Tyr705} is involved in the genesis of the diffuse type of gastric cancer. Its activation through phosphorylation could be blocked at the level of Tyr705, with consequences upon the cell phenotype.

CONCLUSION

In the case of gastric cancer, the morphological features of the cells help us to identify some of the molecules involved in carcinogenesis. The immunohistochemical comparison between the intestinal and the diffuse types has shown a significant difference for pStat3^{Tyr705}, E-cadherin, alpha and beta-catenin, suggesting the key role of EMT in carcinogenesis, so that targeting these key molecules involved in this process could be a therapeutic alternative.

Conflicts of interest. The authors report no potential conflict of interest.

Authors' contribution: S.S., R.B., F. Bibeau, F. Borini, M.P. and C.T. performed the experiments. S.S. and C.T. wrote the manuscript. J.-C.S. read the final version of the manuscript and approved the submission. He also offered financial support for this study.

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