2 遺伝子検査

◎遺伝性腫瘍の遺伝子検査は保険適用となっているものはなく，すべて自費診療か，あるいは研究として行われている．主な遺伝性腫瘍症候群の発端者および家系の臨床的特徴と原因遺伝子を表Ⅱ-8に示す．

表Ⅱ-8 遺伝性乳癌の原因遺伝子と臨床的特徴

病型	発端者および家系の特徴	乳癌以外の腫瘍	原因遺伝子
遺伝性乳癌卵巣癌(HBOC)	・若年発症(50歳未満)，(異時性)両側性乳癌 ・*BRCA1*より高齢で発症，ホルモン受容体陽性が多い(*BRCA2* > *BRCA1*) ・triple negative乳癌(TNBC)(*BRCA1* > *BRCA2*) ・男性乳癌(*BRCA2* > *BRCA1*)	・若年発症(50歳未満)乳癌，(異時性)両側性乳癌 ・漿液性卵巣癌，卵管癌，腹膜癌等のMuller氏管由来腫瘍(*BRCA1* > *BRCA2*) ・前立腺癌(*BRCA2* > *BRCA1*) ・膵癌(*BRCA2* > *BRCA1*)	*BRCA1*・*BRCA2* フルシークエンス検査を最初に実施，陰性の場合，本人希望によりMLPA*法を実施
Li-Fraumeni症候群(LFS)	若年発症乳癌(平均発症年齢33歳)	骨肉腫，軟部組織肉腫，脳腫瘍，副腎皮質癌などの小児癌あるいは胃癌，肺癌等の成人発症の固形腫瘍	*TP53* *CHK2* フルシークエンス検査
Cowden病(CS)	乳癌，甲状腺癌，子宮癌(体癌および頸癌)，消化管ポリポーシス，大腸癌，血管腫，皮膚症状，咽頭乳頭腫		*PTEN* フルシークエンス検査，陰性の場合にMLPA*法
Peutz-Jeghers症候群(PJS)	乳癌，子宮頸癌(悪性腺腫)，卵巣癌(性索間質性腫瘍)，消化管過誤腫		*STK11* フルシークエンス検査，陰性の場合にMLPA*法
遺伝性びまん性胃癌(HDGC)	乳腺小葉癌，スキルス胃癌		*CDH1* フルシークエンス検査，陰性の場合にMLPA*法
*ATM*遺伝子のヘテロ保因者	毛細血管拡張性運動失調症(ataxia-telangiectasia)の原因遺伝子である*ATM*遺伝子のヘテロ保因者，乳癌の放射線治療の際に皮膚障害等を発症することがある．		*ATM* フルシークエンス検査，陰性の場合にMLPA*法
その他，NCCNガイドラインで推奨されている遺伝子パネル	*BRCA1/2*遺伝子検査陰性例および家族歴から一種類以上の症候群が疑われる症例が適応とされる．遺伝子検査ラボにより違いがあるが，上記の*TP53*, *CHEK2*, *PTEN*, *STK11*, *CDH1*, *ATM*に右記の16個の遺伝子を加えた22種類の遺伝子が，次世代シークエンサー(NGS)解析で行われる新規遺伝子検査パネルとして紹介されている． 検査の限界として，意義不明の遺伝子変異variants of unknown significance (VUS)が認められる頻度が不明，ほとんどの遺伝子で関連するリスクが不明であり，遺伝子変異陽性例のリスク管理について明確なガイドラインがないものもある．臨床的有用性に関するデータは限られ，かつ複雑なため，遺伝性腫瘍の専門医にコンサルテーションが必要． (NCCN Clinical Practice Guideline in Oncoogy (NCCN Guidelines) Genetic / Familial High-risk Assessment：Breast and Ovarian Version1.)		*BARD1* *BRIP* *CHEK1* *MLH1* *MSH2* *MSH6* *MUTYH* *MRE11A* *NBN* *PALB2* *PMS2* *PTEN* *RAD50* *RAD51B* *RAD51C* *RAD51D*
多因子遺伝に関わるSNPマーカー	ゲノムワイド相関解析(GWAS)で乳癌リスクが高いことが報告されている一塩基多型(SNP)マーカーの遺伝型の組み合わせによって乳癌罹患リスクを予測できる．*BRCA1/2*遺伝子変異陽性のHBOC症例の浸透率の評価にも有用と報告されているが，日本人での検証は不十分． (Pharoah PD, et al：Nat Genet. 31 (1)：33-36, 2002.)		*FGFR2* *TNRC9* *MAP3K* *LSP1* *rs13281615* *rs13387042* *CASP8*

＊：MLPA：multiplex-ligation dependent probe amplification

〔菅野康吉〕

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Case Report

Cytological Features of a Variant NUT Midline Carcinoma of the Lung Harboring the *NSD3-NUT* Fusion Gene: A Case Report and Literature Review

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Background. Nuclear protein in testis (NUT) midline carcinoma (NMC) is a very rare and aggressive malignancy. In more than two-thirds of these NMC cases, a fusion between *NUT* and *BRD4* or *BRD3* has been documented; other variants are rare. The cytology of NMC itself has been sparsely documented and that of variant NMC has never been reported. *Case Presentation.* A 36-year-old woman was admitted because of a rapidly progressing lung tumor with metastases to the breast and bone. We recently reported this patient as the first case of a variant NMC of the lung harboring an *NSD3-NUT* fusion, based on immunohistochemical and genetic analyses. Cytological material was available for the present review. A highly cellular smear contained a predominantly noncohesive pattern of monomorphic cells with diameters 2–2.5 times greater than those of small lymphocytes, with a round-to-oval nucleus, slightly irregular nuclear contours, variably prominent nucleoli, scant cytoplasm, and identifiable mitotic figures. Foci of stratification and overt pearl formation, including a dyskeratocyte, were occasionally observed. The necrotic background contained naked nuclei, karyorrhectic debris, apoptotic cells, and macrophages phagocytizing karyorrhectic debris; nuclear crushing was noted. *Conclusion.* The cytological features of a variant NMC of the lung are described for the first time.

1. Introduction

Nuclear protein in testis (NUT) midline carcinoma (NMC) is a recently recognized entity that is characterized by undifferentiated morphological features and immunoreactivity to NUT [1]. This disease is a very rare [2–4] and aggressive [3, 4] malignancy that most often occurs in the midline of the body, including the head and neck and the mediastinum [2, 4]. Currently, the diagnosis of NMC depends on the identification of a rearrangement involving the *NUT* locus at 15q14 that generates a specific fusion transcript with a member of the bromodomain-containing protein (BRD) family, such as *BRD4* located on chromosome 19p13.1. In more than two-thirds of NMC cases, a gene fusion between *NUT* and *BRD4* or *BRD3* has been documented [2, 5–7]; other variant fusions are rare [6]. Recently, we described a variant NMC in which an *NSD3- (nuclear receptor binding SET domain 3-) NUT* rearrangement was identified in the primary tissue using 5′-rapid amplification of the cDNA end (RACE); the fusion was validated using fluorescence *in situ* hybridization (FISH) [8].

Little information is available on the cytological features of NMC. Three reports have described the cytological features of 4 common NMC cases [9, 10] and another NMC case in which the gene rearrangement was not analyzed [11], and no information on the cytology of variant NMC is presently

available. We herein describe, for the first time, the cytological features of a variant NMC of the lung harboring an *NSD3-NUT* fusion gene.

2. Clinical Summary

A 36-year-old woman sought medical advice because of a cough accompanied by wheezing with a 2-month duration. An enhanced computed tomography scan performed at the time of hospitalization revealed a mass (75 × 38 × 35 mm in size) in the left lung that extended to the middle mediastinum; metastatic lesions in the liver, breast, bones, and lymph nodes were also detected [8]. A transbronchial biopsy (TBB) of the lung tumor and an endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) of a lower mediastinal lymph node with lung tumor involvement were performed.

3. Materials and Methods

The aspiration material obtained from the lymph node was separated into two parts; its small portion was available for cytological investigation. The specimen was air-dried and Hemacolor-stained (MERCK, Darmstadt, Germany) at bedside according to the manufacturer's instructions and a portion was fixed in 95% ethanol and stained with Papanicolaou as ordinary methods. The larger part of the aspiration material obtained from the lymph node and the biopsy material taken from the lung tumor were immediately immersed in 20% buffered neutral formalin, fixed overnight, and embedded in paraffin. These specimens were then sectioned and used for hematoxylin-eosin staining, immunohistochemistry with antibodies for EMA (DAKO, Glostrup, Denmark), p63 (Santa Cruz Biotechnology, Dallas, TX, USA), cytokeratin AE1/AE3 (DAKO), cytokeratin CAM 5.2 (Becton, Dickinson and Company, CA, USA), CD138 (DAKO), vimentin (DAKO), and others, and FISH, as reported previously [8].

3.1. Cytological Findings. An overview of the Papanicolaou-stained smear showed the specimen to be highly cellular with loosely cohesive cells and/or isolated cells (Figure 1). The cells were 2–2.5 times greater in diameter than that of a small lymphocyte. The nuclei were round to oval in shape and had slightly irregular contours, with one or more prominent nucleoli (Figure 1). The chromatin was hyperchromatic and finely granular in most of the cells or vesicular in occasional cells. The main cells, which had scant cytoplasm and an indistinct cell border, often formed loosely cohesive clusters (Figure 2). These clusters were in contact with foci of stratification (Figure 2), which consisted of occasional cells with a clear cell border and moderately delicate cytoplasm, often with cytoplasmic coarse vacuoles (Figure 3, arrowhead) that were negative for epithelial mucin. In addition, a few cells showed an overt pearl formation, including a dyskeratocyte (Figure 3, arrow), whereas occasional small apoptotic cells with orange G-colored cytoplasm were scattered throughout the specimen. A glandular structure was not observed.

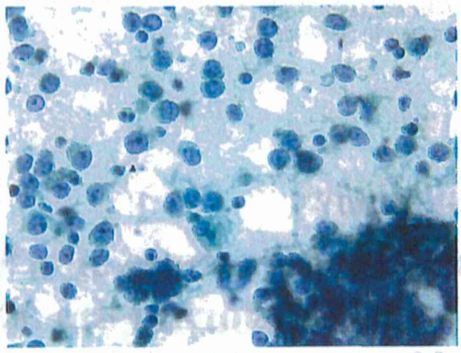

Figure 1: Papanicolaou-stained smear showing a high cellularity with loosely cohesive cells or isolated cells. The cells were 2–2.5 times greater in diameter than that of a small lymphocyte. The nuclei were round to oval in shape with slightly irregular contours and contained one or more prominent nucleoli. The chromatin was hyperchromatic and finely granular.

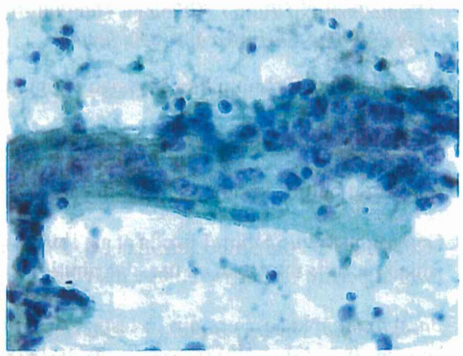

Figure 2: Papanicolaou-stained smear showing loosely cohesive clusters and stratification. The main cells exhibited scant cytoplasm and an indistinct cell border, forming loosely cohesive clusters (right side) that were in contact with foci of stratification (center-left side), which consisted of occasional cells with a clear cell border and moderately delicate cytoplasm.

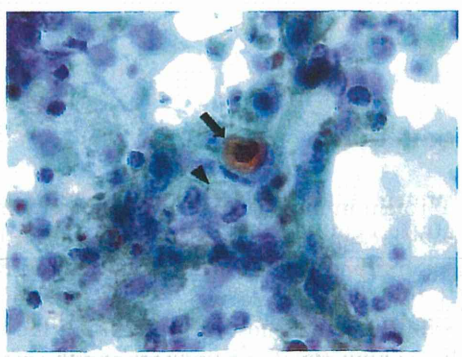

Figure 3: Papanicolaou-stained smear showing pearl formation. A few cells showed overt pearl formation, including a dyskeratocyte (arrow), implying keratinization. Occasional cells with moderately delicate cytoplasm and cytoplasmic coarse vacuoles (arrow head) are visible.

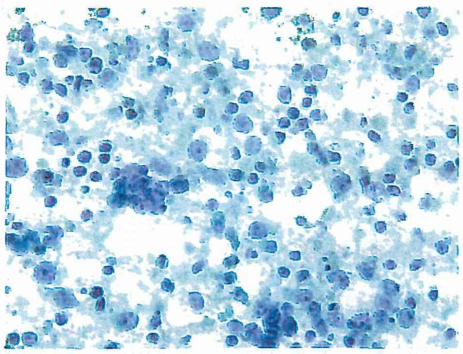

FIGURE 4: Papanicolaou-stained smear showing a necrotic background containing naked nuclei, karyorrhectic debris, apoptotic cells, and macrophages phagocytizing karyorrhectic debris.

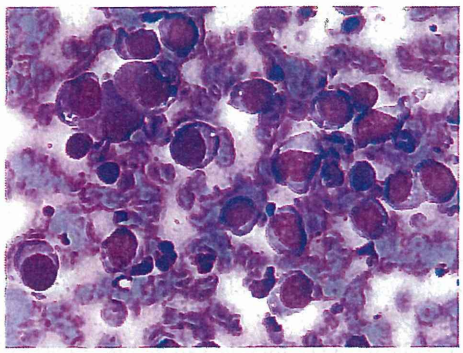

FIGURE 5: Hemacolor-stained smear showing cells bearing basophilic cytoplasm focally with several cytoplasmic fine vacuoles.

Pleomorphism of the cells was not prominent. Mitotic figures were often identified. The necrotic background contained naked nuclei, karyorrhectic debris, apoptotic cells, and macrophages phagocytizing karyorrhectic debris (Figure 4); nuclear crushing was noted.

On the other hand, the Hemacolor-stained smear showed occasional cells bearing delicate basophilic cytoplasm focally with several cytoplasmic fine vacuoles (Figure 5), whereas these cytoplasmic fine vacuoles were not identified in the Papanicolaou-stained smear material.

3.2. Histological, Immunohistological, and FISH Findings. Details of the histological and molecular features of this case have been previously reported [8]. Briefly, only a small amount of biopsy was available for histological investigation, and it revealed an undifferentiated neoplasm with necrosis (Figure 6). In this material, squamous differentiation, which is a possible characteristic of variant NMC, was not apparent.

Immunohistochemical staining demonstrated focal positivity for EMA, p63, cytokeratin AE1/AE3, cytokeratin CAM 5.2, CD138, and vimentin. In addition, a nuclear staining pattern for NUT was evident (Figure 7). Furthermore, FISH analyses revealed an *NSD3-NUT* rearrangement (Figure 8), whereas *BRD3/4-NUT* fusion genes were not identified.

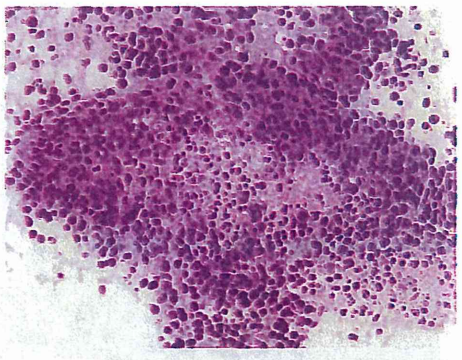

FIGURE 6: Representative histological image showing sheets of undifferentiated malignant cells with focal necrosis.

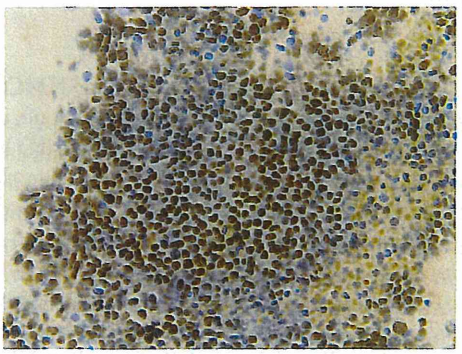

FIGURE 7: Immunohistochemistry showing a nuclear staining pattern for NUT.

3.3. Treatment and Follow-Up. The patient received chemoradiation therapy for 10 months after her diagnosis. However, the patient died with disease progression at 10 months after the diagnosis. Autopsy was not permitted.

4. Discussion

The histological features of NMCs range from entirely undifferentiated carcinomas to carcinomas with prominent squamous differentiation [2, 7, 12–15]. Thus, a diagnosis of NMC based solely on morphology can be difficult. Previous studies have described the cytological features of 4 common NMCs harboring a *BRD3/4-NUT* fusion gene [9, 10] and another NMC case in which the gene rearrangement was not analyzed [11]; the cytological characteristics of these NMCs showed a highly cellular, predominantly noncohesive pattern of relatively small cells with a round nucleus, scant cytoplasm, irregular nuclear contours, variably prominent nucleoli, and identifiable mitotic figures. These findings were also observed in the present variant NMC case. Thus, the previously reported findings imply that the cytological characteristics of NMC are nonspecific and similar to those of undifferentiated carcinoma.

Keratinization has not been identified in previous studies examining the cytology of NMCs [9–11]. In theory, overt keratinization is very important and sometimes pathognomonic for a scrutinized diagnosis of NMC; that is, it is assumed

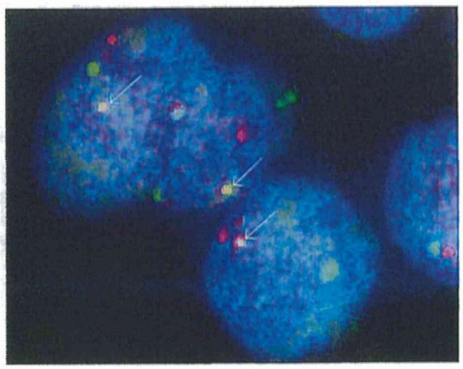

FIGURE 8: A dual-color FISH analysis showing the fusion gene as a single yellow (overlapping) signal (arrows), including a green (*NSD3*) and an orange (*NUT*) signal.

to be a relatively characteristic finding of NMC harboring a *NUT* gene rearrangement involving a gene other than *BRD4* [16]. Keratinization in surgical material, however, is notorious for evading observation because its distribution is often very focal [6, 16], and sampling biases often occur. The cytology of the present NMC variant showed overt pearl formation, including a dyskeratocyte, and the stratification in contact with loosely cohesive clusters; we believe that this finding corresponds to abrupt keratinization, which has been reported as a peculiar characteristic of variant NMC [16] but was not recognized in our corresponding histology specimen [8]. Thus, the combination of cytological and histological findings may help to reveal keratinization concurrent with stratification in undifferentiated carcinoma, which is one clue for a diagnosis of NMC, especially variant NMC, rather than other tumors.

The Hemacolor-stained smear showed occasional cells possessing delicate basophilic cytoplasm with several fine vacuoles, which were not observed in our Papanicolaou-stained smear. In a previous NMC case harboring a *BRD3-NUT* fusion gene, the cells were described as possessing delicate to finely vacuolated cytoplasm, although we could not observe the cytoplasm in detail because of the low magnification of the published figures [10]. On the other hand, these cytoplasmic fine vacuoles were not observed in NMC cases harboring a *BRD4-NUT* fusion gene [9]. Further studies are needed to clarify whether these cytoplasmic fine vacuoles are specific for NMC harboring a fusion gene involving *NUT* and a gene other than *BRD4*.

In conclusion, although the distinction of NMC from other poorly differentiated carcinomas based solely on morphology is difficult, cytological investigation is helpful, especially for identifying abrupt keratinization, which histopathological investigations can miss because of sampling biases. Our experience has shown that the identification of the following clues may suggest a diagnosis of NMC; overt pearl formation including a dyskeratocyte, stratification, and cytoplasmic fine vacuoles, especially in cases where the initial suspected diagnosis was "undifferentiated or poorly differentiated carcinoma with little pleomorphism." Furthermore, the identification of this entity is critical, and immunohistochemistry or FISH studies should be considered for the identification of *NUT* gene rearrangements.

Conflict of Interests

The authors have no conflicts of interest to declare.

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Editorial

TCGA output and practice of gastric cancer

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Any clinical professionals who devote themselves to prevention, diagnosis, therapy, and management of gastric cancer patients are now again facing another achievement by the consortium of The Cancer Genome Atlas (TCGA) (1). In the era of post human genome sequence and massive parallel sequencing technology, every week this kind of huge data draw a transient attention of our colleagues who are very busy with conventional routines. Information like this on the cutting edge of science is not usually related to the action plan next week at the clinic. In a field of lung cancer managements, however, we witnessed the latest fruits of these technologies such as series of discoveries of targetable fused-kinase protein drastically changed clinical practice (2). How influential the results presented here in this article can be to the practice today, tomorrow, and in future? Reasonably, clinicians in any fields of specific organ cancers hope categorization of cancers based on the state-of-the-art technology can specify the fittest therapy in each individual. In the field of gastric cancer research, attempts to delineate genomic characteristics of gastric cancer and to take advantage of these features as potential targets of therapy have been popular in the literatures of the last few years.

For example, Kubo *et al.* reported re-sequencing and copy number analysis of kinases in gastric cancer (3) and Kiyose *et al.* further applied 400 BAC FISH probes on the tissue microarray of 350 gastric cancers, identified several kinase gene amplification, and suggested the assays could be used as companion diagnosis on pathology archives like Hercep Test™ (4). Hillmer *et al.* applied paired-end-tag sequencing approach to four gastric cancers and found structural variations in them (5). Zang *et al.* focused on kinase changes in 14 gastric cancer cell lines (6). Methodologies were various. Deng *et al.* investigated 193 primary gastric tumors by high resolution SNP array and copy number changes in the tumors. Based on the huge mutational information of gastric cancer obtained by massively parallel short read and DNA paired-end tag sequencing, Nagarajan *et al.* tried to classify gastric cancers into two categories; microsatellite instability-positive gastric cancer and TP53-wild type cancer (7). Then Zang *et al.* did exome analysis of 15 cases and disclosed mutations of chromatin modifier genes such as *ARID1A* and cell adhesion molecule such as *FAT4* (8). As to the MSI positive fraction of gastric cancer, Korean researchers extensively clarified mutation profile (9). In the course of rapid popularity of "genome-wide" approaches applied to each cancer case, a peculiar pathological status became clarified such as *GLO* amplification as a new metabolic marker of gastric cancer (10).

In the paper published in September issue of Nature, TCGA reported the landscape of somatic changes in gastric cancer in comprehensive way. The data they showed include mutations per Mb, copy number changes (somatic copy number alteration SCNA), DNA methylation, mRNA expression profile, micro RNA profile analysis, microsatellite instability, Epstein-Bar virus infection status as well as whole genome analysis for identification of structural changes (such as fusion genes) found in gastric cancer. According to the supplementary table of this paper, out of 295 cases, the cases with T1A and T1B are 11 (3.7%) and the T3 cases are about half of the total cases. This fact implies the idea and consequent strategy for gastric cancer therapy generated by this study are mainly applicable to T3 andT4, an advanced stage gastric cancer, some of which are inoperable. For example, the managements widely recommended in Japan, that is, detection of gastric cancer at early stage by intensive surveillance and endoscopical submucosal dissections (*Figure 1*) for nearly asymptomatic subjects (covered by government-based health insurance),

Figure 1 An example of the Hematoxylin Eosin stained section of T1A tumor obtained by endoscopical submucosal dissection (Hamamatsu University School of Medicine). The study includes only one case of this stage. The comprehensive genetic study of gastric cancer in this stage is still not available.

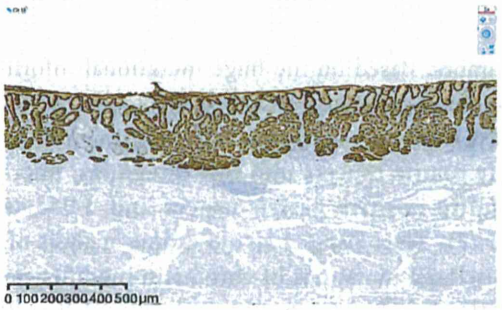

Figure 2 Immunohistochemistry of claudin 18 in human stomach. Monoclonal antibody to CLDN18, a member of tetraspan transmembrane protein of tight junction (Proteintech, Chicago, IL) was used in 200× dilution.

are out of the scope of this costly analysis and therapeutic plan based on it they envisaged here.

The tremendous data set published by TCGA suggested four categories of gastric cancer: (I) EBV positive cases; (II) microsatellite instability (MSI) positive cases; (III) chromosomal instability (CIN) type; (IV) genomically-stable type (near-diploid type). The hallmarks of these four groups can be said in another way: (I) hypermethylation type; (II) KRAS, PTEN, PIK3CA mutations; (III) kinase receptor amplifications; (IV) diffuse type with RHOA mutation, respectively.

For the last two decades, the above four aspects of gastric cancer have been repeatedly investigated both sporadically and systematically in various scale of projects including the very recent studies by two group which identified RHOA mutation (11,12). The genes where mutations were more frequently found than *RHOA* are *TP53*, *CDH1*, *SMAD4*, and *PIK3CA* which are consistent with the previous reports, and *ARID1A*, *KRAS*, *MUC6*, and *APC* followed. The authors highlighted the *PI3KCA* mutations, extreme methylation, and amplifications of *JAK2*, *PD-L1*, and *PDL2* in EBV positive category. As for fusion genes, transcripts involving *CLDN18i*, which is specifically expressed in gastric epithelium (*Figure 2*) were detected. Its partners were ARHGAP family genes. The genes involved in this and related pathways have been investigated for years such as involvement of ARHGEF 6 (beta-PIX) and ARHGEF (alpha-PIX) by the researchers of cell signaling (13-16) and the involvement of ARHGAP 6 and 26 in this TCGA paper are mechanistically understandable, especially considering these were found in diffuse type, notoriously invasive subtype of gastric cancers. The finding that the 5' side of fusion transcript is CLDN18, a claudin specifically expressed in the stomach reminds us that *SLC34A2* specifically expressed in type II alveolar cell of the lung has been found as a component of fusion transcript in some of lung cancers (17,18).

Based on these data, the authors encourage the readers, and probably themselves, by pointing out that the signaling molecules above-mentioned could be targetable. The involvement of PD-1 and 2, immune checkpoint inhibitors, in EBV related gastric cancer is remarkable considering these molecules are enthusiastically promoted as targets of immunotherapy (especially in malignant melanoma) (19). Obviously the practical feasibility of the management of gastric cancer based on the proposals of this paper warrants further applied and translational researches and assessments by several sectors including academics, industries, health insurance companies, and attending doctors.

The other point to bewilder the practical pathologists is histological sub-classification shown in the **supplemetary table (1)**. Sub-classification of gastric cancer ranged from Lauren dichotomy (actually this paper adopts a trichotomy including mixed type) to the Japanese classification systems (http://www.jgca.jp/pdf/JGCA_Jpn_Classification_3rd_Eng.pdf, 2011) which morphologically scrutinize very minute attributes up to the level where it sometimes suffers from the Galapagos Syndrome—it has evolved separately from the rest of the world. WHO system would be a wise and modest way when describing the statistics. The most pathologists, however, are very familiar with the morphological heterogeneity in single tumor in advanced stage gastric cancer especially where several blocks (five and more, sometimes 30 to 50) are routinely made for pathological investigation. As expected, the histological sub-classification itself was not related to molecular signature shown here. Thus the cancer, a real challenge we should treat may evade "individualized" therapeutic strategy this

ambitious presentation proposes. On the other hand, in the next stage, the application of the tour de force genetic analyses to the initial stage of gastric carcinogenesis will further provide efficient predictive and preventive measures of this ominous cancer.

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Gastric cancer in young vs old Romanian patients: immunoprofile with emphasis on maspin and mena protein reactivity

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Increasing number of early-onset gastric carcinomas (GCs) and controversial results regarding the differences among young and older patients with this type of cancer are the reasons why correlation of clinicopathological factors with molecular markers is necessary. The aim of our study was to compare the demographic, clinical and immunohistochemical (IHC) aspects in Romanian patients with GC diagnosed below and above 45 years old. In 191 samples provided from patients with GC, the clinicopathological parameters were correlated with a panel of 15 antibodies: E-cadherin, HER-2, VEGF, CD31, CD105, COX-2, maspin, bax, bcl-2, p53, Ki67, MLH-1, MSH-2, mena protein and vimentin. Compared to the conventional cases, GCs diagnosed below 45 years old were more frequent located at the gastroesophageal junction and presented a higher percentage of lymph node metastases. The diffuse type E-cadherin/mena/p53/Ki67/bax-negative cases that displayed nuclear maspin positivity were also more frequently in younger patients. The intestinal type early-onset GCs were the most angiogenic ones, the apoptotic rate being lower than in the intestinal type GCs of the aged. Compared to the conventional cases, in the early-onset GCs the nuclear maspin-mediated antiproliferative activity is more intense in diffuse type while the mena-dependent tumor cell proliferation is more characteristic for intestinal type GCs.

Key words: Gastric cancer; maspin; mena protein; angiogenesis; microsatellite status.

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Despite well-conducted national screening programs, gastric cancer (GC) still remains the fifth most common malignancy and the third leading cause of cancer death worldwide (1). In countries with poorly developed or absence of programs related to early diagnosis, screening and surveillance, the incidence is even higher; it represents the fourth most common malignancy and the second leading cause of cancer-related deaths (2, 3).

In Romania, the national screening program was not yet organized and absence of a cancer registry does not allow a proper assessment of survival data. However, Romania is a country with relatively high incidence of GC (about 16.66/100.000); this tumor represents the fourth place in males and the fifth in females, being the second cause of cancer-related deaths (4, 5). Although the mortality rate slightly declined in the last decade, the 3-year- and 5-year overall survival rates still remain at about 30% and 15%, respectively, and is even lower in elderly males (4, 5).

Moreover, in most of the countries, the screening program starts at the age of 40, except for the hereditary cases, in which the eligible families are recommended to begin screening at the age of 18 and even 16 (3, 4, 6, 7). Due to delayed diagnosis (7), the pathomechanism and behavior of early-

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onset GCs is not well understood either its characteristics compared with the conventional cases diagnosed in patients older than 40–45 years old. In most of the studies, the aggressive behavior of early-onset carcinomas was mainly sustained by the lower rate of survival in young patients compared to the elderly patients (7), although this was not always significant (3). Moreover, due to association of other comorbidities in older patients (3), the survival rate is not a reliable factor to compare the aggressiveness between cases diagnosed in younger and older patients. Taking into account the absence of symptoms in early stages, the diagnosis of most of the cases in advanced stages in both age-related groups and different risk of relapse and overall survival for patients with the same stage (8), more comprehensive studies are necessary to elucidate the clinicopathological and molecular aspects of early-onset cases compared to the conventional ones.

To characterize the early-onset GCs compared to the cases diagnosed in the elderly, we tried to analyze the classic clinicopathological factors but also the immunoprofile of these cases based on a large panel of antibodies, the real importance of some of them for GCs being not yet reported in the literature. To asses this goal, we used a panel of 15 antibodies. They were related to the rate of tumor proliferation (Ki67), tumor aggressiveness (p53), neoangiogenesis (vascular endothelial growth factor (VEGF-A), COX-2, CD31, CD105), apoptosis (bcl-2, bax), the microsatellite status (MLH-1, MSH-2), and the epithelial-mesenchymal transition (vimentin). The antibody anti-HER-2 that is usually used as predictive value for targeted therapy with trastuzumab was also used. The immunohistochemical (IHC) expression of mena protein, a cytoskeletal protein (9) about no data was reported in the PubMed system, referring to the GC, was also studied and correlated with the cell–cell adhesion molecule E-cadherin. The last marker that was used to complete the whole immune-picture was maspin, a serine protease that seems to play antiangiogenic, proapoptotic, and antiproliferative roles in other cancers such as breast, prostate, or colorectal carcinomas but few aspects were proved to be concordant in the 27 papers published to date regarding its expression in gastric lesions; one of the reasons could be the particular subcellular pattern of this marker that can be expressed in cytoplasm, nuclei, or both components of the tumor cells (10). No such comprehensive study was yet performed to compare the clinicopathological and IHC particularities of GC in younger and older patients. No studies about mena expression in GC have yet to be published.

MATERIALS AND METHODS

Patients and tissue samples

The clinicopathological features of GCs were investigated in 191 consecutive cases that underwent curative resection (partial or total gastrectomy) from 2006 to 2013 at Clinical County Hospital of Tirgu-Mures, Romania. In most of the cases (95.8%), D1 lymph node dissection was performed. No preoperative chemo- or radiotherapy was used in any of the cases. The study was conducted at the Department of Pathology of University of Medicine and Pharmacy of Tirgu-Mures, Romania, and partial handling of obtained data was performed at the Department of Tumor Pathology of Hamamatsu University School of Medicine, Japan. The head of the Department of Pathology of the University of Medicine and Pharmacy of Tirgu-Mures, Romania, approved the processing of the cases. Informed consent of patients was obtained for this study.

For comparative purposes, the 191 cases were divided in two groups: 37 patients aged 45 years and younger (group 1) and 154 patients who were older than 45 years old (group 2). Besides demographic data, type of surgical intervention, and the peritumoral presence, or absence of metaplasia, the following parameters of the tumors were evaluated in both groups: anatomic localization, macroscopic and microscopic features, and the tumor stage (pT, pN, pM), according to the 7th edition of the American Joint Committee on Cancer staging criteria (11). Macroscopically, according to the Borman's classification, the tumors were categorized in the following four groups (11, 12): I. Polypoid; II. Ulcerated; III. Ulcero-infiltrative; IV. Diffusely infiltrative. Microscopically, using the criteria indicated by the World Health Organization combined with the Lauren's classification, the cases were clustered in two categories (diffuse- and intestinal type) divided into the following six sub-categories (12, 13): diffuse type carcinomas (divided into poorly cohesive and signet ring cell adenocarcinomas), and intestinal type carcinomas (divided into four groups: very well-differentiated, well/moderate-differentiated (G1/G2), poorly differentiated adenocarcinomas (G3), and papillary carcinomas). No mucinous intestinal type or even mixed-type carcinomas have been identified in this cohort. The very well-differentiated adenocarcinomas were defined, according to Yao et al., as tumors composed of small-sized highly differentiated tubular structures with mild nuclear atypia that have the ability to invade the gastric wall in a diffuse manner (14).

To establish the tumor stage, the presence/absence of intestinal metaplasia, and to perform the microscopically classification, all slides stained with hematoxylin and eosin were re-examined by two experienced pathologists.

Immunohistochemistry – methods and antibodies

In each of the 191 cases the immunostains were performed on 5-μm thick formalin-fixed paraffin-embedded tissues using the 60 holes Quick-Ray tissue array (TMA) blocks (IHC World, Woodstock, MD, USA). For a proper reliability, three punches (2 mm-sized core biopsy) from each of the cases (tumor tissue) have been used. In non-reliable stains, the reactions were repeated using the classical

slides. A panel of 15 antibodies was used; their characteristics are presented in Table 1. Novolink Polymer detection system (Novocastra, NewCastle Upon Tyne, UK) was used for processing of the cases and DAB (diaminobenzidine) solution (Novocastra) for developing. Antigen retrieval was done using a high pressure-cooker. Counterstaining was done with Mayer's hematoxylin (Novocastra). For negative controls, incubation was done with omission of specific antibodies.

Immunohistochemistry – scoring

Two pathologists (GS, JI) quantified independently the expression of all the 15 antibodies without knowledge of other clinicopathological parameters. The IHC assessment was performed with Nikon 800E optical microscope, with digital photo camera. The cut-off point for membrane expression of E-cadherin and cytoplasmic positivity for VEGF-A, COX-2, bax, bcl-2, mena protein, and vimentin-positivity was defined as 10% (8). HER-2 positivity was also evaluated in the cell membrane, using the criteria previously described in literature as specific for gastric cancer (15). A positive reaction for p53 and Ki67 proteins and also for the antibodies anti-MLH-1 and MSH-2 was defined as nuclear staining in >10% of the tumor cell nuclei.

The microvessel density (MVD) was counted with both CD31 and CD105 using digital pictures and the image analysis software ImageJ (NIH). The MVD was recorded by counting the positive vessels in the highly vascularized ('hot-spot') areas, at 200× high-power fields. We batch measured the percentage of positive endothelial area vs total area of the microscopic field. The ulcerated and inflammatory areas were not taken into account for assessment of angiogenesis.

Maspin immunoreactivity was separately analyzed in nuclei and cytoplasm of the tumor cells based on the previously described methods, the cut-off value of positivity was 10% (10). The cases were divided into the following four groups: negative cases, cytoplasmic predominance, nuclear predominance, and mixed expression of maspin (cytoplasmic and nuclear expression).

Statistical analysis

A Microsoft Excel table was created containing clinicopathological and immunohistochemical data and was used to calculate the frequency distributions of specific parameters. The results were further evaluated using the GraphPad InStat 3 statistical software. A p-value <0.05 with 95% confidence interval was considered statistically significant. The data were presented using descriptive analysis; the frequencies and percentages specific for the analyzed parameters and means and standard deviations were used for continuous variables. The *t*-test, chi square test, and the contingency tables, Fischer's test, Mann–Whitney test, and One way ANOVA test were used for univariate analysis. For the IHC assessment, the interobserver reliability was calculated by Cohen's kappa-type test (10); in cases with low agreement, re-evaluation was performed to establish a final consensus score. Multivariate analysis involved the binary logistic regression model.

RESULTS

Clinicopathological features

The median age of the 191 patients was 63.98 ± 14.83 years (range, 21–98 years), without an age-dependent difference regarding the male:female ratio. In group 1, the median age was 37.59 ± 6.52 years (range, 21–45 years), while group 2 presented a median age of 68.88 ± 8.98 years (range, 46–98 years).

Most of the cases diagnosed in patients in group 1 were located in the proximal area and/or greater curvature while the distal stomach and smaller curvature were more involved in cases diagnosed in patients in group 2. The ulcerated aspect of the tumors was predominated in both groups, although the diffuse type GCs, especially those with signet ring features were predominated in group 1, without associated metaplasia in most of them. Independent of the grade of differentiation, the intestinal type GCs and also tumor-associated metaplasia were especially diagnosed in patients in group 2. The papillary carcinomas mostly occurred in group 1. There were not significant correlations between the macroscopically and microscopically tumor type ($p = 0.21$) even between anatomic tumor localization and other clinicopathological parameters (Table 2).

As for the tumor stage, in most of the cases the clusters of tumor cells crossed the muscularis propria; a slightly lower deepness of infiltration (pT stage) was seen in group 1.

A significant difference was related to the lymph node metastases, a highest percentage of node-positive cases being noticed in patients in group 1 (97.3%) compared to group 2 (78.6%). No differences were seen between the two groups regarding the distant metastases (Table 2).

The very well-differentiated adenocarcinomas presented same clinicopathological and IHC features as the classic well/moderately differentiated adenocarcinomas.

Correlation of the immunoprofile with clinicopathological data

For each of the 15 antibody assessment, except CD31 and CD105, the interobserver concordance of the results was 91% and the mean κ value was >0.68. In the non-concordant cases, the final consensus score obtained after re-evaluation was used. For CD31 and CD105, the NIH's Image System was used to calculate the MVD; no consensus was necessary.

The statistical correlations proved some different patterns of the examined antibodies displayed by the tumor cells of younger vs older patients. Both

Table 1. The main characteristics of the antibodies used for immunostains

Antibody (company)	Clone	Dilution	Antigen retrieval	Positive control
Maspin (Novocastra, Newcastle-upon-Tyne, UK))	EAW24	1:25	Incubation with citrate buffer (pH 6.0) – 60 min at 100 °C	• external – prostate and myoepithelial cells (breast) • internal – NS
Mena (BD Biosciences, New Jersey, NJ, USA)	Isotope mouseIgA clone 21	1:25	Incubation with citrate buffer (pH 6.0) – 60 min at 100 °C	• external – previous positive colorectal carcinomas • internal – smooth muscle fibers
p53 (LabVision, Fremont, CA, USA)	DO-7	Ready to use	Incubation with high pH-solution (pH 10.0) – 30 min at 100 °C	• external – normal colon mucosa – basal cells • internal – NS
Ki67 (LabVision)	SP6	Ready to use	Incubation with citrate buffer (pH 6.0) – 30 min at 100 °C	• external – tonsils • internal – normal gastric mucosa
Bcl-2 (LabVision)	100/D5	1:50	Incubation with citrate buffer (pH 6.0) – 30 min at 100 °C	• external – tonsil and lymph node • internal – lymphocytes
Bax (LabVision)	2D2	1:25	Incubation with citrate buffer (pH 6.0) – 30 min at 100 °C	• external – Hodgkin's lymphoma • internal – NS
CD31/PECAM-1 (LabVision)	JC/70A	1:25	Incubation with high pH-solution (pH 10.0) – 30 min at 100 °C	• external – placenta • internal – blood vessels – endothelial cells
CD105/endoglin (Novocastra)	Mouse monoclonal	1:25	Incubation with citrate buffer (pH 6.0) – 60 min at 100 °C	• external – tonsils • internal – NS
VEGF-A (Ab-7) (LabVision)	VG1	1:50	Incubation with high pH-solution (pH 10.0) – 30 min at 100 °C	• external – renal tubes • internal – normal mature vessels – endothelial cells
COX-2 (Novocastra)	Monoclonal	1:100	Incubation with citrate buffer (pH 6.0) – 60 min at 100 °C	• external – brain • internal – lymphocytes
HER-2 (Dako, Glostrup, Denmark)	5A2 cerbB2-oncoprotein	1:1000	Incubation with high pH-solution (pH 10.0) – 30 min at 100 °C	• external – ductal epithelial cells of the breast • internal – NS
MLH-1 (Novocastra)	ES05	1:100	Incubation with citrate buffer (pH 6.0) – 30 min at 100 °C	• external – colon – normal mucosa • internal – lymphocytes
MSH-2 (Novocastra)	25D12	1:50	Incubation with citrate buffer (pH 6.0) – 60 min at 100 °C	• external – lymph node – germinal centers • internal – lymphocytes
E-cadherin (Dako)	NCH-38	1:50	Incubation with citrate buffer (pH 6.0) – 30 min at 100 °C	• external – breast ductal carcinoma • internal – normal gastric mucosa
Vimentin (Dako)	V9	1:800	Incubation with high pH-solution (pH 10.0) – 30 min at 100 °C	• external – tonsil • internal – lymphocytes

NS, non-specific internal control; for a proper reliability, simultaneous external control was used.

E-cadherin and mena protein expression was significantly lost in patients in group 1, especially within the diffuse type GCs. Their expression was also lost in about one-quarter of the cases with diffuse-phenotype diagnosed in patients in group 2 (Fig. 1). As for the intestinal type carcinomas, their immunoreactivity was correlated with the grade of differentiation; absence of both E-cadherin and mena was rather specific for poorly differentiated adenocarcinomas (Tables 3 and 4).

The immunoexpression of p53 was more frequently lost in group 1; in group 2 the index of positivity

Table 2. Analysis of clinicopathological features of patients with gastric cancer related to the patient's age

Parameter	≤45 years (n = 37 cases)		>45 years (n = 154 cases)		p-value
	Number	%	Number	%	
Gender					
Male	23	62.2	106	68.8	0.37
Female	14	37.8	48	31.1	
Ratio M:F	1.6:1		2.2:1		
Tumor location					
Antrum	10	27.05	64	41.6	**0.0009**
Smaller curvature	10	27.05	55	35.7	
Body	4	10.8	20	13	
GEJ	8	21.6	9	5.8	
Greater curvature	5	13.5	6	3.9	
Type of surgical procedure					
Total gastrectomy	21	56.8	67	43.4	0.09
Partial gastrectomy	16	43.2	87	56.5	
Macroscopic type					
I	1	2.7	9	5.8	0.16
II	5	13.5	9	5.8	
III	24	64.9	115	74.7	
IV	7	18.9	21	13.7	
Histologic type					
Diffuse poorly cohesive	14	37.9	33	21.5	**<0.0001**
Diffuse-signet ring cell	8	21.6	5	3.2	
Intestinal very well diff.	3	8.1	14	9.1	
Intestinal G1 + G2	5	13.5	55	35.7	
Intestinal G3	5	13.5	43	27.9	
Intestinal papillary	2	5.4	4	2.6	
pT stage – tumor infiltration					
1	1	2.7	9	5.8	0.06
2	1	2.7	11	7.1	
3	8	21.6	50	32.5	
4	27	73	84	54.6	
pN stage – lymph nodes					
0	1	2.7	33	21.4	**0.0004**
1	5	13.5	24	15.6	
2	7	18.9	31	20.1	
3	24	64.9	66	42.9	
pM stage – distant metastases					
0	31	83.8	124	80.5	0.71
1	6	16.2	30	19.5	
Metaplasia					
Yes	3	8.1	92	59.7	**<0.0001**
No	34	91.9	62	40.3	

Bold indicate statistically significant values.

was mostly higher than 50%. This significant negativity was especially characteristic for the diffuse type GCs of group 1 (Fig. 1) that also presented Ki67 negativity.

Independent of the patient's age and other clinicopathological characteristics, loss of E-cadherin expression was correlated with p53 negativity ($p = 0.0001$). Compared to the E-cadherin positive cases, those that were negative for E-cadherin were also more frequently negative for Ki67 (91.3% vs 50%), HER-2 (94.4% vs 80.4%), and mena (76% vs 29.6%).

Microsatellite instability-high level (MSI-H), indicated by double negativity of MLH-1 and MLH-2, was not implicated in the pathogenesis of the early-onset GCs (Table 3). The HER-2 positivity was more frequently seen in the well/moderately differentiated adenocarcinomas and papillary carcinomas, independent of the patients' age (Tables 3 and 4).

As for the angiogenesis, the mean MVD counted with CD31 was 4.02 ± 1.65 (range, 0.97–12.86). The mean MVD counted with CD105 was 3.09 ± 1.83 (range, 0.20–10.49). The cases with MVD ≤3.74 (CD31) and 3.24 (CD105), respectively, were considered as having low MVD, the other ones were considered as highly angiogenic tumors.

Without differences between the two groups, COX-2 intensity was directly correlated with the MVD counted with CD31 ($p = 0.01$) but not with

Table 3. Analysis of immunohistochemical features of gastric cancer cells related to the patient's age

IHC antibody	≤45 years (n = 37 cases)		>45 years (n = 154 cases)		p-value
	Number	%	Number	%	
E-cadherin					
Negative	10	27	19	12.3	**0.01**
Positive	27	73	135	87.7	
HER-2					
Negative	33	89.2	126	81.8	0.22
Positive +++	4	10.8	28	18.2	
VEGF					
Negative	5	13.5	48	31.2	**0.003**
Positive	32	86.5	106	68.8	
CD31					
Low	18	48.7	80	51.9	0.77
High	19	51.3	74	48.1	
CD105					
Low	7	18.9	90	58.4	**< 0.0001**
High	30	81.1	64	41.6	
COX-2					
Negative	11	29.7	36	23.4	0.33
Positive	26	70.3	118	76.6	
Maspin					
Negative	5	13.5	36	23.4	**0.001**
Cytoplasm	12	32.5	74	48.1	
Mixed	16	43.2	41	26.6	
Nuclei	4	10.8	3	1.9	
BAX					
Negative	25	67.6	27	17.5	**< 0.0001**
Positive	12	32.4	127	82.5	
Bcl-2					
Negative	35	94.6	147	95.5	1.00
Positive	2	5.4	7	4.5	
P53					
Negative (<5%)	22	59.5	66	42.9	**0.03**
Low (<50%)	9	24.3	43	27.9	
High (>50%)	6	16.2	45	29.2	
Ki67					
Negative (<5%)	23	62.2	79	51.3	0.26
Low (<50%)	7	18.9	41	26.6	
High (>50%)	7	18.9	34	22.1	
MLH-1					
Negative	3	8.1	34	22.1	**0.009**
Positive	34	91.9	120	77.9	
MSH-2					
Negative	–	–	7	4.5	0.06
Positive	37	100	147	95.5	
MLH-1 + MSH-2 negative (MSI-H)	–	–		4.5	
Mena					
Negative	19	51.3	48	31.2	**0.006**
Positive	18	48.7	106	68.8	
Vimentin					
Negative	26	70.3	143	92.9	**< 0.0001**
Positive	11	29.7	11	7.1	

IHC, immunohistochemical; MSI-H, microsatelitte instability-high level.
Bold indicate statistically significant values.

CD105 ($p = 0.13$) and was higher in the intestinal type GCs. The VEGF intensity was directly correlated with the MVD counted with CD105 ($p = 0.003$) but not with CD105 ($p = 0.12$) and was higher in group 1 than group 2. In both groups, the intestinal-type was more angiogenic than the diffuse type (Tables 3 and 4; Figs 1 and 2).

Although maspin expression was more frequent lost in group 2, the difference was non-significant ($p = 0.14$). Independent of the tumor type, the

Table 4. Analysis of the immunoprofile of gastric cancer cells related to the patient's age and the Lauren's histological type

IHC antibody	≤45 years (n = 37 cases)		>45 years (n = 154 cases)		p-value
	A. Diffuse type n = 21 (55.8%)	B. Intestinal type n = 16 (44.2%)	C. Diffuse type n = 38 (24.9%)	D. Intestinal type n = 116 (75.1%)	
E-cadherin					
Negative	10 (47.6%)	–	11 (28.9%)	9 (7.8%)	A vs B < 0.0001
Positive	11 (52.4%)	16 (100%)	27 (71.1%)	107 (92.2%)	A vs C = 0.008
					C vs D = 0.0002
					B vs D = 0.006
HER-2					
Negative	20 (95.2%)	13 (81.3%)	36 (94.7%)	90 (77.6%)	A vs B = 0.003
Positive +++	1 (4.8%)	3 (18.7%)	2 (5.3%)	26 (22.4)	A vs C = 1.25
					C vs D = 0.0007
					B vs D = 0.72
VEGF					
Negative	3 (14.3%)	–	11 (28.9%)	34 (29.3%)	A vs B < 0.0001
Positive	18 (85.7%)	16 (100%)	27 (71.1%)	82 (70.7%)	A vs C = 0.01
					C vs D = 1.12
					B vs D < 0.0001
CD31					
Low	12 (57.1%)	6 (37.5%)	25 (65.8%)	57 (49.2%)	A vs B = 0.007
High	9 (42.9%)	10 (62.5%)	13 (34.2%)	59 (50.8%)	A vs C = 0.24
					C vs D = 0.02
					B vs D = 0.11
CD105					
Low	6 (28.6%)	1 (6.3%)	34 (89.5%)	67 (57.8%)	A vs B < 0.0001
High	15 (71.4%)	15 (93.7%)	4 (10.5%)	49 (42.2%)	A vs C < 0.0001
					C vs D < 0.0001
					B vs D < 0.0001
COX-2					
Negative	8 (38.1%)	2 (12.5%)	14 (36.8%)	21 (18.1%)	A vs B < 0.0001
Positive	13 (61.9%)	14 (87.5%)	24 (63.2%)	95 (81.9%)	A vs C = 1
					C vs D = 0.004
					B vs D = 0.32
Maspin					
Negative	5 (23.8%)	1 (6.3%)	8 (21.1%)	23 (19.8%)	A vs B < 0.0001
Cytoplasm	5 (23.8%)	9 (56.2%)	10 (26.3%)	63 (54.3%)	A vs C = 0.47
Mixed	8 (38.1%)	5 (31.2%)	17 (44.7%)	4 (3.5%)	C vs D < 0.0001
Nuclei	3 (14.3%)	1 (6.3%)	3 (7.9%)	26 (22.4%)	B vs D < 0.0001
BAX					
Negative	15 (71.4%)	12 (75%)	13 (34.2%)	14 (12.1%)	A vs B = 0.63
Positive	6 (28.6%)	4 (25%)	25 (65.8%)	102 (87.9%)	A vs C < 0.0001
					C vs D = 0.0003
					B vs D < 0.0001
P53					
Negative	15 (71.4%)	9 (56.2%)	16 (42.1%)	51 (44%)	A vs B = 0.03
Positive	6 (28.6%)	7 (43.8%)	22 (57.9%)	65 (56%)	A vs C < 0.0001
					C vs D = 0.88
					B vs D = 0.11
Ki67					
Negative	17 (81%)	5 (31.2%)	25 (65.8%)	66 (56.9%)	A vs B < 0.0001
Positive	4 (19%)	11 (68.8%)	13 (34.2%)	50 (43.1%)	A vs C = 0.02
					C vs D = 0.24
					B vs D = 0.0003
Mena					
Negative	17 (81%)	3 (18.7%)	19 (50%)	29 (25%)	A vs B < 0.0001
Positive	4 (19%)	13 (81.3%)	19 (50%)	87 (75%)	A vs C < 0.0001
					C vs D = 0.0004
					B vs D = 0.39

IHC, immunohistochemical; VEGF, vascular endothelial growth factor.

mixed and nuclear expression was more specific for group 1 while the cytoplasmic expression rather occurred in group 2. Comparative to the intestinal type GC, the diffuse type mostly displayed nuclear pattern in younger and mixed pattern in older. Independent of the patient's age, the intestinal type GCs showed more frequent cytoplasmic expression (Tables 3 and 4; Figs 1 and 2).

The multivariate analysis showed that, in the diffuse type GCs of the pateins in group 1, Maspin nuclear expression was predominantly associated with negativity for bax, Ki67, p53, and mena protein, and a lower MVD, while the VEGF expression was inconstantly positive (HR, 0.77; 95% CI, 0.43–1.03). The patients in group 2, with diffuse type GCs displayed more frequently mixed maspin positivity, correlated with negativity for bax and Mena, with a lower MVD but with positivity for VEGF, Ki67, and p53 (HR, 0.74; 95% CI, 0.56–1.21). In both groups, maspin cytoplasmic predominance displayed by the intestinal type GCs was associated with positivity for p53, Ki67, E-cadherin, mena protein, and VEGF and a higher MVD (HR 0.95; 95% CI, 0.75–1.24). In these cases, the bax negativity was predominant in group 1 while the tumor cells for intestinal type GCs diagnosed in patients in group 2 were mostly bax positive (Tables 3 and 4; Figs 1 and 2).

DISCUSSION

Being a highly heterogenic tumor, surgical resection is considered the mainstay for therapy of GC (8) and the targeted therapy is only based on the HER-2 expression and amplification of *HER-2* gene, respectively. To date, several markers have been investigated in GC samples but single molecular markers have been usually used, the results being controversial. In this study, we tried to perform a molecular characterization of GC in young and elderly patients, in contrast to other studies, and to outline the first steps in age-dependent molecular classification of GC, based on own data and a review of the literature.

In this material, the male predominance was seen in both younger and older patients, in other previously reported studies the females were the ones being affected at younger ages than the males, without influence on the overall survival rate (3, 7, 16). Although increasing number of GCs located in the proximal stomach was reported to increase in the late decades, this growing rate was especially observed in our material as specific for the Romanian younger patients and not for older patients. The poorly cohesive carcinomas were more predominated in other reported data (3, 16); the Romanian younger patients also presented a highly percentage of cases with diffuse pattern. The predominance of diffuse pattern in the younger group, in concordance with a lower rate of intestinal metaplasia, can be partially explained by the tumor location: gastroesophageal junction in younger and distal part (probably *H. Pylori*-related) in older (7).

As for the tumor stage, although most of the cases are diagnosed in late pathologic pT stages, independent of the age of patient, this material showed that the node-positive cases are more frequent in younger patients. In our experience we also noticed that, in the last year, the number of

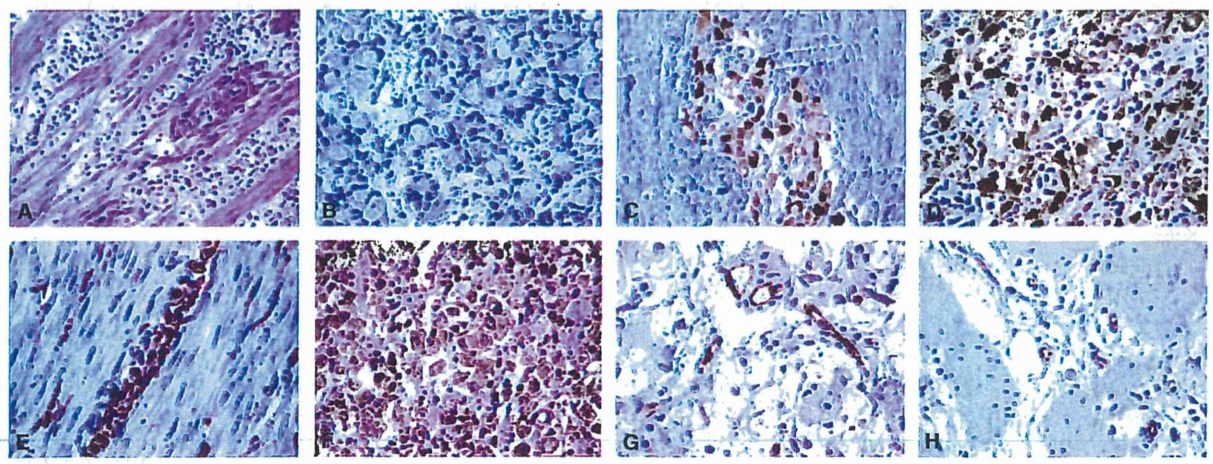

Fig. 1. The diffuse type gastric cancer does not present immunoreactivity for mena protein (A) and p53 (B), display a nuclear (C) or mixed nuclear-cytoplasmic maspin expression (D), can adopt epithelial-mesenchymal transition revealed by vimentin positivity (E), has a diffuse low-intensity VEGF expression (F) and a relatively low microvessel density in older (G,H). Ob. 20×.

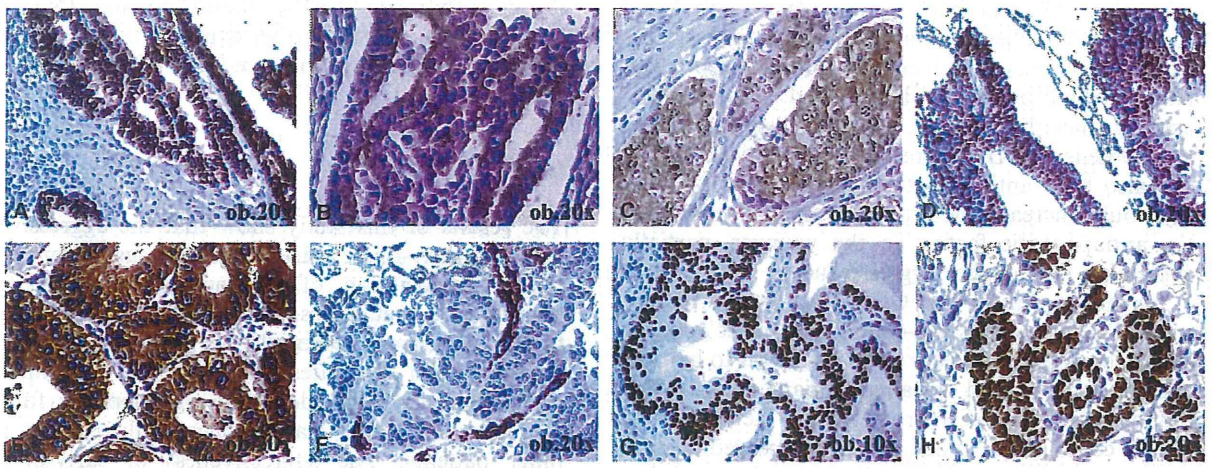

Fig. 2. Intestinal type gastric cancer display intense positivity for E-cadherin (A) and mena protein (B), cytoplasmic predominance of maspin (C), bax (D) and VEGF positivity (E), well-developed CD105-positive vessels (F), and high proliferative rate showed by Ki67 (G) and high p53 index (H).

cases with multifocal carcinomas increased, distant and skip metastases being even present in early GC of Romanian patients with both diffuse- and intestinal type (S. Gurzu, I. Jung, S. Haruhiko & T. Bara, personal communication). On the basis of these facts, we consider that the therapeutic guidelines should be changed and adapted on the patient's age, total gastrectomy with D2 lymph node dissection being advisable to be used on young patients, even in East European countries, independent of the tumor stage, especially in biopsy-proven diffuse-architecture (diffuse type and poorly differentiated adenocarcinomas). Classic surgery should be performed in diffuse type GC, even in early stages, rather then submucosal endoscopic resection that should be limited to the dysplastic cases. To sustain this fact, we are also basing it on the recently proved fact that, even in mucosal GC, the risk of lymph node metastases is higher in diffuse type and poorly differentiated adenocarcinomas than in the well and moderately differentiate intestinal type adenocarcinoma, especially in young patients (16).

Although pathologic stage remains the gold standard of prediction relapses, it is rather based on the number of lymph node metastases than the depth of infiltration (3, 16) and is not influenced by the macro- and microscopical type of tumors either by the grade of differentiation, tumor location and demographic data (age and gender) (8). However, it was suggested that the expression of p53 should supplement the current staging system in terms of relapse prediction (8), without references at the early-onset and conventional cases. The p53 is an independent predictive factor of relapse (8) but not of survival rate (17). Its negativity is correlated with a lower rate of recurrences in both advanced stages III and IV and the positivity is also related to a less effectiveness of 5-Fluorouracil and cisplatin chemotherapy in these cases (8). On the basis of the predominant negativity of the diffuse type carcinomas of the young patients that was confirmed by other authors (18), we consider that, in early-onset GCs, this index could rather be used as an indicator of the response to chemotherapy, if this is indeed proved to be effective, but not as a prognostic marker. It seems that the aggressiveness of diffuse type GCs diagnosed below 45 years is not related to the p53 index even to the Ki67 index of tumor proliferation; the *p53* gene alterations is rather seen in the intestinal type GCs, independent of the patient's age (18).

The angiogenesis was more expressed in the early-onset intestinal type GCs, proving that they could respond to the antiangiogenic therapy that did not seem to be efficient in the diffuse type GCs that had a lower MVD. On the other hand, the early-onset intestinal type GCs could have a more aggressive behavior, being proved that a high VEGF tissue level and/or a high CD105 MVD are indicators of locoregional and hematogenous recurrences/metastases, including the peritoneal ones (19, 20).

The maspin expression and its multivariate correlations can add arguments for different pathways involved in the carcinogenesis of early-onset and conventional-GCs but also the Lauren's diffuse- and intestinal types, independent of the patient's age. First of all, the intestinal type GCs, independent of the patient's age, proved to have a maspin cytoplasmic predominance, this subcellular pattern having a pro-angiogenic effect on the tumor cells. The increased rate of tumor cell proliferation could

be explained by the intense synchronous activity of the cell–cell adhesion marker E-cadherin and the cytoskeleton marker mena. However, the older patients had a proper bax-related pro-apoptotic activity than the younger, while the younger had a more intense VEGF expression. Because the VEGF can also have antiapoptotic function (21), it probably could increase the resistance of tumor cells at the antiapoptotic factors in the younger, partially explaining their higher aggressiveness.

As for the diffuse type GCs, it seems that its maspin-related genesis is age-dependent. In the younger group, the maspin nuclear pattern showed to have an antiproliferative and impaired antiangiogenic effect but does not act as a proapoptotic factor for the tumor cells. In the older group, the mixed maspin expression showed to inhibit the neoangiogenesis but does not have proapoptotic even antiproliferative effect. Independent of the patient's age, in the diffuse type GCs, the cytoskeleton protein mena seems to not be involved in the tumor cell proliferation.

These correlations suggest that, in early-onset diffuse type GCs, nuclear maspin acts as a tumor suppressor protein with antiproliferative and minimal antiangiogenic functions, while the mixed maspin expression, predominated in the older group, exert only an antiangiogenic effect. In intestinal type cancer, independent of the patient's age, cytoplasmic maspin predominance is not enough for even the antiangiogenic and/or antiproliferative effect, while the bax-dependent proapoptotic function is kept only in the older group. In these cases, the Mena protein and E-cadherin are responsible by a higher proliferative rate of tumor cells.

Experimentally, it was proved that, in GC cell lines, maspin downregulation accelerated the cell cycle progression but its upregulation retarding cell proliferation (22). The present study adds that nuclear subcellular pattern is essential for its antiproliferative function, it being especially present in the early-onset diffuse type GCs.

As we already mentioned, the E-cadherin and mena expression seems to be lost more frequently in the diffuse type GCs, especially at the younger ages, but the poorly differentiated tubular adenocarcinomas diagnosed at older ages can also lose their immunoreactivity. However, the relapse rate is not related to E-cadherin negativity (8, 17) rather, it being used to indicate a hereditary component. We suggest that E-cadherin should be tested in daily diagnosis, in patients with diffuse architecture (diffuse type and poorly differentiated adenocarcinomas) independent by the patient's age and, in negative cases, *CDH1* mutations should be tested, the result being very useful for familial screening. Being a cytoskeleton protein, mena activity was significantly related to E-cadherin function. Its gene alterations should be further explored in GCs, especially in patients with hereditary diffuse gastric cancer.

CONCLUSIONS

The results of this study show that the aggressiveness of early-onset gastric carcinomas is mainly due to association of an increased number of lymph node metastases, compared to the conventional carcinomas, especially in diffuse type cases. It seems that the intestinal type GCs are mena and E-cadherin dependent, the proapoptotic function of cytoplasmic maspin being only present in older patients. The aggressiveness of early-onset diffuse type GCs compared to the conventional ones, seems to be determined by the nuclear maspin-mediated activity and not on the increased proliferative rate.

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