

Pathobiological behavior and molecular mechanism of signet ring cell carcinoma and mucinous adenocarcinoma of the stomach: A comparative study

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Supported by the National Natural Science Foundation of China, No.30070845 and No.30371607

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Received: 2003-09-09 **Accepted:** 2003-10-22

Abstract

AIM: To elucidate the distinctive pathobiological behavior between signet ring cell carcinoma (SRC) and mucinous adenocarcinoma of the stomach.

METHODS: Based on the histological growth patterns and cell-functional differentiation classifications of stomach carcinoma, we conducted a series of comparative studies. All paraffin-embedded and frozen blocks were collected from the files of Cancer Institute of China Medical University. On the basis of histopathological observation, we applied enzymatic and mucous histochemistry, immunohistochemistry, flow cytometry (FCM) and molecular biology to compare these two categories of gastric cancers in terms of the DNA ploidy, proliferative kinetics, the expression of gastric carcinoma associated gene product and instabilities of mitochondrial DNA (mtDNA).

RESULTS: Gastric SRC was commonly seen in females below 45 years, mostly presenting diffuse growth and ovary or uterine cervix metastasis. The majority of SRC were absorptive and mucus-producing functional differentiation type (AMPFDT), which growth relied on estrogen. Meanwhile, stomach mucinous adenocarcinomas were mostly observed in males over 50 years, prone to massive growth or nest growth and extensive peritoneal infiltration, showing two categories of cell-functional differentiation types: AMPFDT and mucus-secreting functional differentiation type (MSFDT). Expressions of ER, enzyme c-PDE and 67kDaLN-R in SRC were evidently higher than that in mucinous adenocarcinoma, while expressions of LN, CN-IV, CD44v6, and PTEN protein were obviously lower in SRC than that in mucinous adenocarcinoma ($P < 0.05$). There was no statistic significance in VEGF, ECD and instabilities of mtDNA ($P > 0.05$) between the above two gastric carcinomas.

CONCLUSION: Though SRC and mucinous adenocarcinoma were both characterized by abundant mucus-secretion, they were quite different in morphology, ultrastructure, cell-functional differentiation and protein expression, indicating different mechanisms of carcinogenesis. We concluded that

combining histological growth patterns, cell-functional differentiation types with tumor related markers might be significant in early diagnosis and prognosis assessment for SRC and mucinous adenocarcinoma of the stomach.

Yang XF, Yang L, Mao XY, Wu DY, Zhang SM, Xin Y. Pathobiological behavior and molecular mechanism of signet ring cell carcinoma and mucinous adenocarcinoma of the stomach: A comparative study. *World J Gastroenterol* 2004; 10(5): 750-754

<http://www.wjgnet.com/1007-9327/10/750.asp>

INTRODUCTION

SRC and mucinous adenocarcinoma of the stomach were generally confounded as “mucoïd carcinoma” until 1964 when Zhang *et al* proposed that the so-called “mucoïd carcinoma” included two categories that presented different growth patterns. “Mucoïd carcinoma” was finally divided into mucinous adenocarcinoma and SRC in WHO’s histological classification of the stomach in 1974. At that time, it remained unclear of the different biological behaviors and metastatic or infiltrative characteristics of these two stomach carcinomas.

From 1962, Cancer Institute of China Medical University began a series of studies on histological growth patterns, cell-functional phenotype classifications and infiltrative, metastatic patterns of stomach carcinoma, and a series of comparative studies on SRC and mucinous adenocarcinoma of the stomach were also carried out the following 40 years. We classified functional differentiation types of gastric cancer by histopathological observation, enzymatic and mucous histochemistry, and detected the expressions of related genes with the help of biological techniques. Different biological behaviors of local infiltration and metastasis of SRC and mucinous adenocarcinoma were investigated at morphological, functional and protein levels.

MATERIALS AND METHODS

All paraffin-embedded and frozen blocks were collected from the files of Cancer Institute of China Medical University. On the basis of histopathological observation, AKP, ACP and LAP were detected by enzymatic histochemistry and various kinds of mucin were detected by mucous histochemistry in order to propose a new cell-functional classification of gastric carcinomas. We observed the expression of ER, cPDE, laminin (LN) and its receptor 67kDaLN-R, collagen-IV (CN-IV), CD44v6, VEGF, ECD and PTEN proteins by immunohistochemistry, the variation of DNA ploidy, proliferative kinetics by flow cytometry (FCM), and three adjacent regions of mtDNA (D-loop, tRNA^{phe} and 12S rRNA) were detected for instabilities via PCR amplification followed by direct DNA sequencing and dHPLC. All procedures were done according to references^[1-11].

RESULTS

SRC and mucinous adenocarcinoma were found quite different

in pathological morphology, functional differentiation phenotypes, molecular pathology mechanisms and prognosis as follows.

Morphologic observation

The different growth patterns of mucinous adenocarcinoma and SRC were firstly proposed, following the histological growth classifications of the stomach in 1964. The former presented nest or massive growth, which cancer cells produced and secreted abundant mucus-like substance and then hoarded in the cancerous nest; while the latter presented signet ring cells, widely infiltrated in the stomach wall, accompanied by the formation of “migratory cancerous embolus of lymphatic vessel”. SRC had specific cytogenetics: cancer cells were separate, and presented irregular amebocyte shape. Image of ameboidism was often observed emigrating from lymphatic vessel especially in loose part of the stomach (subserosa and submucosa), which suggested the characteristic of widespread growth and invasion. This image was not shown in mucinous adenocarcinoma of nest or massive growth. Meanwhile, the mesenchymal reaction was also quite different between the above two stomach carcinomas: cancerous foci of mucinous adenocarcinoma was mainly enwrapped by collagen fibers, and the argyrophilia fibers were thick and intensive to bundles, surrounding the outside of the cancerous foci; some transition to collagen fibers and even basement membrane-like structures were shown. There were such lymphoid cells and macrophages as inflammatory reaction in adjacent cancerous foci. However, mesenchyma of SRC was loose and in edema, and argyrophilia fibers were reticularly loose, which suggested that the host

immunological reaction was weaker than that of the mucinous adenocarcinoma and coincident with its diffusely infiltrating growth pattern.

Functional differentiation vs invasion and metastatic characteristics

Functional differentiation investigation showed that more than 80.0% of SRC showed AMPFDT: SRC cells not only produced mucus, but also expressed intestinal enzymes of absorption cell markers (AKP and LAP), ACP, and estrogen receptor (ER) in most cancer cells.

Mucinous adenocarcinoma showed specific functional differentiation: 55.3%(21/38) of them showed single MSFDT; 44.7%(17/38) were AMPFDT. The latter was similar to SRC in biological characteristics, while the former did not express intestinal enzymes of absorption cell markers or ER (Table 1).

Tumor related markers

Expressions of ER, enzyme c-PDE and 67kuN-R were evidently higher in SRC than in mucinous adenocarcinoma, while expressions of LN, CN-IV, CD44v6, and PTEN protein were obviously lower in SRC than those in mucinous adenocarcinoma ($P<0.05$). There was no statistic significance in VEGF, ECD and instabilities of mtDNA ($P>0.05$) between the two gastric carcinomas. In SRC, DNA ploidy was different at different pathologic stages: the elevated contents of DNA, polyploid and heteroploid were often seen in early SRC; while the content of DNA was lower and diploid or subdiploid were often presented in advanced SRC. Polyploid and heteroploid were often observed in mucinous adenocarcinoma (Table 1).

Table 1 The comparison of pathobiologic characteristics between SRC and mucinous adenocarcinoma

Pathobiologic characteristics	SRC (AMPFDT)	Mucinous adenocarcinoma	
		AMPFDT	MSFDT
Onset age	Mostly below 50	Same as the left	Mostly over 50 ^[1,2]
Sex	more in female	Same as the left	More in male ^[1,2]
Infiltrative depth	16.7% (2/12) passing through the serosa		70.0% (7/10) passing through the serosa ^[1,2]
Growth pattern	Diffuse growth 100.0%(12/12)		Diffuse growth 40.0%(4/10), massive growth 60.0%(6/10) ^[1,2]
Metastasis rate of lymph node	66.7% (8/12)		80.0%(8/10) ^[1,2]
Distant metastasis	Often to ovary and uterus, sometimes to bones, marrow, bladder, blepharon or skin of lower extremities	Same as the left	Prone to peritoneal extensive infiltration ^[1,2]
Tumor related markers			
ER	Positive rate 75.0%(9/12)		Positive rate 10.0%(1/10) ^[1,2]
c-PDE	Positive rate 80.0%(4/5)		Positive rate 20.0%(1/5) ^[3]
LN	Few, positive rate 25.0%(2/8)		BM*-like line structure was seen frequently, positive rate 83.3%(5/6) ^[4]
67KDa LN-R	Positive rate 75.0%(15/20)		Positive rate 25.0%(1/4) ^[5]
Collagen IV	CN-IV positive fragments or particles only, positive rate 87.5%(7/8)		BM*-like line structure was seen frequently
CD44V ₆	Positive rate 11.8%(2/17)		Positive rate 100.0%(6/6) ^[4]
PTEN	Low expression (25.0%, 7/21)		Positive rate 62.5%(5/8) ^[6]
VEGF	Positive rate 90.0%(9/10)		High expression (60.0%) 6/10 ^[8]
ECD	Negative expression 85.0% (17/20)		Positive rate 100.0%(7/7) ^[7]
DNA ploidy	Negative expression 85.0% (17/20)		Negative rate 75.0%(3/4) ^[9]
	Polyploid and heteroploid were often seen in early stage, while diploid and hypodiploid were often seen in advanced stage		Polyploid and heteroploid were often observed ^[10]
mtDNA variation	66.7%(2/3), para-cancerous		66.7%(2/3), para-cancerous
D-loop region	tissue 33.3%(1/3)		tissue 66.7%(2/3)
12S rRNA	33.3%(1/3)		66.7%(2/3) ^[11]
Ultrastructure	More mucous secretion. Few desmosome junctions and gap junctions between cells.		Plenty of mucous secretion. Several desmosome junctions and gap junctions between cells
5 year survival rate	15.9%		19.4%

*Basement membrane.

DISCUSSION

The survival rate of both SRC and mucinous adenocarcinoma was extremely low. According to the National Cooperation Group of Stomach Carcinoma, the 5-year survival rate of SRC and mucinous adenocarcinoma of the stomach was only 15.9% and 19.4% respectively mainly because most clinical cases of SRC or mucinous adenocarcinomas were moderate to advanced cancer accompanied with extensive infiltration and metastasis. Metastasis, especially unmanageable metastasis to the remote important viscera was the primary cause of patients' death. Research findings of more than 40 years revealed different infiltrative and metastatic mechanisms of SRC and mucinous adenocarcinoma at morphological, functional and protein levels, based on histological growth patterns and cell functional differentiation phenotypes.

The structural basis of different growth patterns of SRC and mucinous adenocarcinoma was demonstrated by electron microscopic observation and mucus histochemistry by Wang *et al* (Cell Biology Institute of China Medical University) and other Chinese scholars^[12]. Under electron microscope, SRC was lack of free ribosomes but rich in rough endoplasmic reticulum (RER), lysosomes, mucus granules, and Golgi complex presented cystiform dilatation, which suggested SRC cells had a strong capability of protein and mucus synthesis. In addition, there were few microvilli on the surface of SRC cell membrane, desmosome junction and gap junction ultimately vanished, the interspaces of cancer cells were enlarged, which suggested that adhesive ability of cancer cells decreased and detachment was easy among cells. Histochemical observation found that these changes were accompanied by releasing of multiple sialomucin, sulfuric acid mucopolysaccharide (AMP), and polysaccharide hydrolases such as acid phosphatase (ACP). These characteristics contributed to the potential of dissolving surrounding tissues, and strong infiltration and metastasis. Nevertheless, cancer cells of mucinous adenocarcinoma were rich in free ribosomes, with scattered RER, and fewer Golgi complex and lysosomes than that of SRC. Further, different from SRC, microvilli and part of desmosome junction and gap junction were shown on the surface of cell membrane. All above characteristics suggested mucinous adenocarcinoma had a stronger adhesive ability than SRC, which partly explained their different growth patterns.

In 1979, Zhang *et al* firstly reported that diffusely growing SRC could show retrograde metastasis from the stomach to cervix and parametrium via "migratory cancerous embolus"^[13], which raised two questions at the same time: Why was it often seen that SRC or poorly differentiated adenocarcinoma and undifferentiated carcinoma containing signet ring cell metastasized to cervix and parametrium, but it was rarely seen in mucinous adenocarcinoma? Was there any difference in cell-functional differentiation and associated molecular pathological characteristics apart from morphologic differences?

The authors detected cell-functional differentiation of gastric cancer cells by enzymatic, mucus histochemistry and immunohistochemistry, proposed a new concept of cell-functional classification of stomach carcinoma, and found that the carcinoma of different functional types were quite different in local infiltration and metastasis to remote viscera^[1,2]. Based on this study, we extensively investigated the hypostases of different biological behaviors of the above two carcinomas^[2]. According to cell-functional differentiation of cancer cells, more than 80.0% of SRC showed AMPFDT, which suggested it was a specific carcinoma cell type with disturbed cell-functional differentiation, possessing both absorptive and mucus-producing functions, and its growth and infiltration relied on ER. SRC not only produced mucus, but also expressed small intestine absorptive cell marker enzymes and ACP. Mucus-producing functional differentiation and the release

of ACP in SRC contributed to hydrolyze mesenchyma surrounding the cancerous foci and invade into normal tissue; and absorptive functional differentiation contributed to absorb nutrition from the host, which accelerated its malignant proliferation. SRC cells customarily expressed ER, which suggested that its growth relied on the existence of estrogen. The reason why SRC was often observed in the female of the premenopause and prone to metastasize to ovary or uterine cervix was its high affinity to organs with a high level of estrogen such as ovary. During the metastasis from the stomach to ovary, "ER (on cancer cells)-estrogen (in the ovary)" affinity linkage might play an important role. Our results suggested that under the prerequisite of hematogenous metastasis, lymphatic metastasis or implantation metastasis to the ovary or cervix, cancer cells with high activities of ER had specific affinity and adaptability to organs rich in estrogen and thus could easily metastasize to the ovary or cervix^[2].

Mucinous adenocarcinoma exhibited two functional classifications as AMPFDT and MSFDT. The biological behaviors and part of molecular pathological characteristics of the former were similar to that of SRC; but the latter did not express small intestine absorptive cell marker enzymes, which disabled its absorptive activity, decreased absorption of enough nutrition and restrained the growth and spread. It was probably one reason why mucinous adenocarcinoma was prone to massive or nest growth, and thus exhibited better prognosis than SRC. These two functional classifications of mucinous adenocarcinoma were quite different in onset age, serosa infiltration, dependence on estrogen (ER expression), *etc.* It was suggested that mucinous adenocarcinoma had the heterogeneity both in cell-functional differentiation and biological feature. So special attention should be paid to cell-functional differentiation characteristics in judging malignant biological behaviors and metastatic patterns of mucinous adenocarcinoma.

In order to explore the difference of molecular mechanisms between the above two gastric carcinomas, we also designed a series of molecular pathological markers ranging from cell proliferation and differentiation, extracellular matrix, tumor angiogenesis, tumor suppressor genes, *ect.* Cyclic nucleotide acid phosphodiesterase (cPDE) is a key enzyme degrading cyclic adenosine phosphate (cAMP) and cyclic guanosine phosphate (cGMP), thereby influencing cell proliferation and differentiation. This study revealed that SRC exhibited higher activities of cAMP-PDE and cGMP-PDE than mucinous adenocarcinoma, and it was concluded that SRC cells had stronger abilities of synthesizing and secreting cPDE than mucinous adenocarcinoma. And cAMP-PDE was a reliable enzymatic marker to estimate malignant degree and prognosis of the above two gastric carcinomas^[3]. LN was the main ingredient of basement membrane. The studies found stomach cancer cells could not only destroy basement membrane but also unceasingly synthesize the ingredient of its basement membrane. Mucinous adenocarcinoma cells had the capacity of synthesizing, and excreting LN out of the cells, which formed a line-like structure similar to basement membrane. This might be one of the reasons why mucinous adenocarcinoma often grew in a nest or massive shape. SRC synthesized very few LN and there were only a few LN particles in extracellular matrix. This might be related to some characteristics of SRC, such as infiltrative growth and high invasiveness^[4]. The quantity and expression intensity of 67KDa LN-R influenced the infiltrative migration of malignant tumor cells. Acting as a kind of LN receptor, 67KDa LN-R usually was involved in the identification of ECM and information convection. Increased expression of 67KDa LN-R in tumor cells would benefit adhesiveness and infiltration. Among the different histological types of gastric cancer, the expression of 67KDa LN-R was

highest in SRC (75.0%, 15/20), and lowest in mucinous adenocarcinoma (25.0%, 1/4), which might be in relation to their different abilities of infiltration and metastasis^[5]. The change of LN expression level was closely correlated to the pathological behaviors of gastric cancer, and could be used as an objective marker to assess the growth ability and the tendency of hematogenous metastasis between the above two gastric cancers. CN-IV was expressed in the above two gastric cancers, but the expression pattern was quite different^[4]. In mucinous adenocarcinoma, the basement membrane-like structure containing CN-IV was seen clearly. However, only linear fragments or positive particles containing CN-IV were seen in SRC. The findings suggested that the above two gastric cancers had different capacities of synthesizing and secreting CN-IV hydrolase. CN-IV hydrolase was strong in the interstitial of SRC, and hydrolyzed CN-IV into discontinuous fragments or particles, which led to the infiltrative growth of cancer cells. The expression of CN-IV in gastric cancer presented negative correlation with its infiltrative growth ability, and could be used as one of the markers to evaluate the poor prognosis of the above two gastric cancers^[4].

CD44v6 took part in specific adhesion processes between cells or cells and matrix. Our results^[6,14] showed CD44v6 had a close relation to metastatic potential of gastric cancer cells and the poor prognosis of patients. It was found that the protein expression of CD44v6 was quite higher in mucinous adenocarcinoma than in SRC. This was probably due to the crypt formation and nest or massive growth pattern of mucinous adenocarcinoma. VEGF is correlated with tumor angiogenesis. A lot of evidence showed that VEGF greatly increased the probability of tumor metastasis by accelerating blood vessel growth. But between the above two gastric cancers, the difference of VEGF expression was not significant^[7]. Up to now, PTEN/MMAC1/TEP1 gene is the first tumor suppressor gene that was proved to have phosphatase activity. Using immunohistochemistry, we found the expression of PTEN protein in SRC was significantly lower than in mucinous adenocarcinoma. The expression of PTEN protein presented negative correlation with gastric cancer's pathological grade and prognosis, which indicated that PTEN gene mutation or inactivation might be one of the reasons for the poorer prognosis of SRC compared with mucinous adenocarcinoma^[8]. ECD is a kind of transmembrane glycoprotein, which induced the adhesion of homogenous cells. The dysfunction of ECD made the adhesion of homogenous cells impossible. Thus, cancer cells tended to separate from each other and metastasize. ECD presented negative expression in 85% (17/20) of SRC and 75% (3/4) of mucinous adenocarcinoma. So ECD could be used as one of the markers to evaluate the poor prognosis of the above two gastric cancers^[9].

There were some studies on tumor related markers from other aspects about the above two gastric cancers. For example, the expression of GST- π was lower in SRC (50.0%, 4/8) than that in mucinous adenocarcinoma (66.7%, 6/9), and it was related to differentiation degree of cancer cells. The expression of GST- π in well differentiated cells was higher, and it was closely correlated with carcinogenesis and progress of gastric cancer, but there was no significance^[15]. S-Tn antigen is a prosoma of saliva acidulated T antigen, and belongs to the embryo or differentiated antigen. It might take part in the early malignant transformation of gastric cancer. It has been reported that the expression of S-Tn antigen in SRC was highest among different histological types of gastric cancers^[16], while the mechanism is still unknown.

DNA aneuploidy of tumor cells is one of its malignant characteristics. DNA aneuploidy of gastric cancer is closely related to the remote organic metastasis, especially to the hematogenous metastasis^[10]. There were a lot of polyploid and

heteroploid cells in early SRC, while most of advanced SRC showed diploid or hypodiploid cells. It suggested that the reduction of DNA content in advanced SRC cells was probably related to the accumulation and crushing of mucus. SRC had the tendency to infiltrate into submucosa in early stage, and this behavior was related to the DNA heteroploidy of cancer cells. Detection of DNA ploidy, especially early detection would help to estimate the malignant level and poor prognosis of the above two gastric cancers.

Recently, researchers have paid more attention to the relationship between the damage of mtDNA and carcinogenesis. mtDNA is a 16 569-bp double stranded, closed circular molecule, which encodes polypeptides participating in oxidative phosphorylation and synthesis of ATP. Compared to nuclear DNA, mtDNA is more susceptible to damage, because of the lack of protective action by histones and the limited capacity of damage repairing system as in yeast and most nuclear genomes. Mitochondrial D-loop and hypervariable region suffered the attack of ROS easily. Chomyn *et al* considered that mtDNA was not necessary to the start-up of necrosis, but it influenced the speed of necrosis because the decrease or damage of mtDNA would result in the increase of ROS^[17]. Our study found that there was no statistic significance in instabilities in D-loop region between gastric cancerous and para-cancerous tissues. There was a significant correlation between differentiation degree of gastric cancer and variant frequencies of 12S rRNA-tRNA^{phe}. The poorly differentiated gastric cancers were more prone to 12S rRNA-tRNA^{phe} variations. But there was no significance between SRC and mucinous adenocarcinoma^[11]. This result needs to be proved by larger sample studies. The relationship between mtDNA and gastric cancer would further be clarified.

There were several molecular biology studies about gastric SRC and mucinous adenocarcinoma. MSI is a frequent genetic change in gastric cancer. Up to now, the average rate of MSI was 33.9% among 29 sites that have been detected, and phenotypes of MSI were different in gastric cancers due to their different pathological types^[18,19]. MSI-positive frequency of SRC was significantly higher than mucinous adenocarcinoma of the stomach, which indicated that the occurrence and progress of the above two gastric cancers probably had different molecular mechanisms. Loss of heterozygous (LOH) is one of MSI phenotypes. The fractional allelic loss (FAL) (the odds of LOH positive marker number and MSI marker number) was related to infiltrative ability of gastric cancer cells and different growth patterns of gastric cancer. Our studies suggested that there might be different allelic deletion between SRC and mucinous adenocarcinoma, which might clarify that the different heredity phenotypes and biological behaviors of the above two gastric cancers were closely correlated with MSI and LOH. We plan on marking MSI and screening LOH site so as to discover minimal deletion fragment nearby these sites by molecular biology and LCM technique. This would lead us to detect some unknown TSGs and study the molecular biological mechanisms of different biological behaviors of the above two gastric cancers.

In conclusion, from the view of histological growth patterns and cell-functional differentiation types of gastric cancer, we investigated various kinds of pathobiological behaviors of gastric SRC and mucinous adenocarcinoma. On the basis of this, we studied on tumor related markers for early diagnosis and prognosis evaluation of gastric cancer, so to instruct clinical surgical treatment scientifically and effectively, and improve survival rate and survival quality. In our cancer institute, the 5 year survival rate of gastric cancer has risen from 19.6% to 58.5%, approaching international advanced level^[20]. Along with the preliminary accomplishment of human genome project, the studies on tumor genomics are advancing with

every passing day. A lot of tumor related genes will be found, and their roles need to be clarified in occurrence, progress and prognosis of the above two gastric cancers. Meanwhile, little literature has been reported on the morphogenetic or histogenetic mechanisms and early diagnostic markers of gastric SRC and mucinous adenocarcinoma, and there is still much dispute in this field. All these problems need further research.

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Edited by Zhu LH Proofread by Xu FM