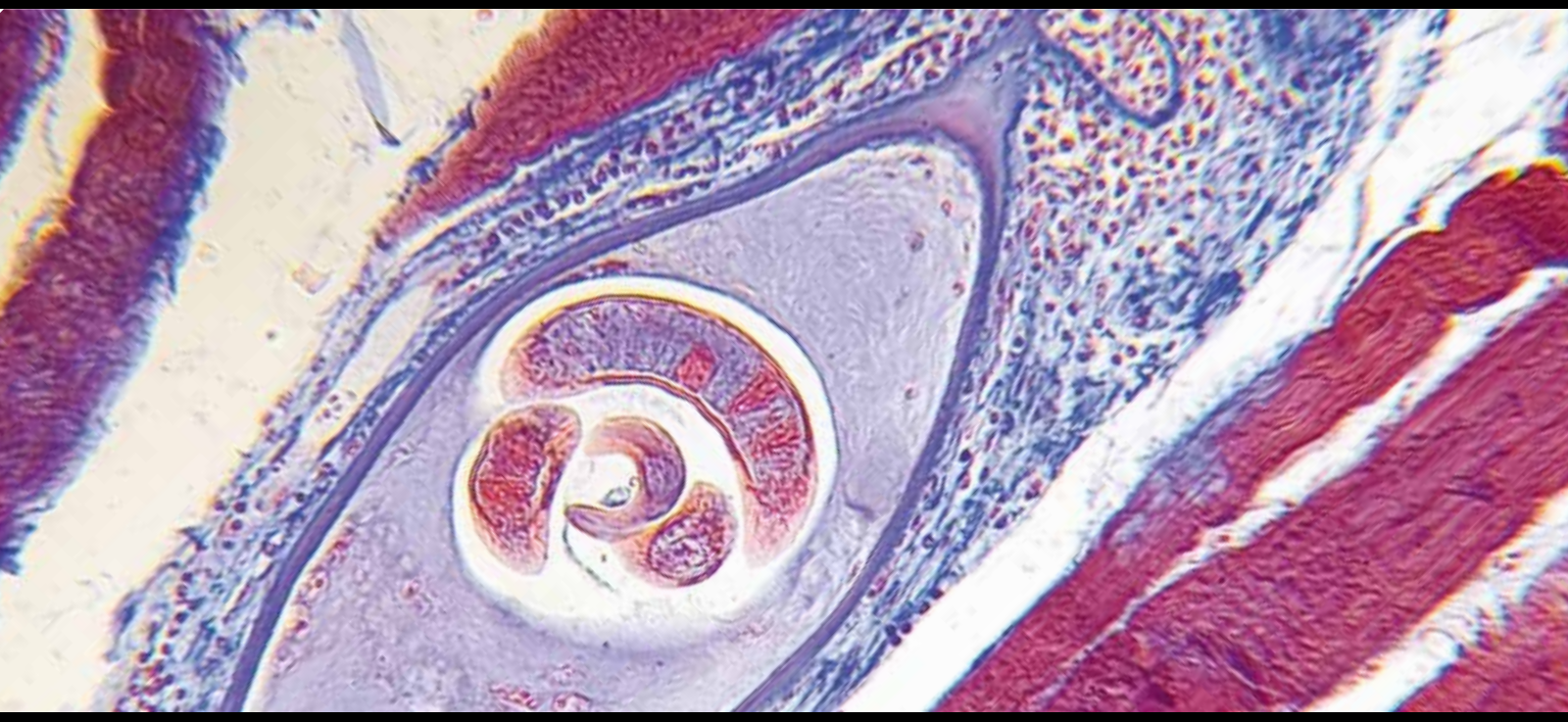
Gastroenterology Research and Practice

Gastrointestinal Cancer Metastasis

Guest Editors: Jin-Lian Chen, Richard Ricachenevsky Gurski, Keiichi Takahashi, and Roland Andersson



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Editorial Board

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Editorial

Gastrointestinal Cancer Metastasis

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It is well acknowledged that the poor prognosis of gastrointestinal cancer is largely due to metastasis, which is the biological hallmark of malignant tumors. Metastasis is considered to be a multistep process in which malignant tumor cells spread inconsecutively from original site to distant organs. As for cancers of gastrointestinal tract, the common target site for metastasis is liver, lymph nodes, peritoneal, and, subsequently, lung and other sites of the body, which in single or as a whole, is the major cause of gastrointestinal cancer-related deaths.

Therefore, the early diagnosis and effective treatment of gastrointestinal cancer metastasis is of vital importance, which may lead to substantial elongation of life expectancy of gastrointestinal cancer patients. In order to make further progressions in early diagnosis and intervention, the following three directions should be put due emphases on in future research.

Firstly, the cellular and molecular mechanisms of gastrointestinal cancer metastasis should be clarified, such as the mechanisms of local angiogenesis, the heterogeneity of cancer cells, the cross-talk and cascade of signaling transduction systems, the decreased expression of cell adhesion molecules such as E-cadherin, and so on. To elucidate these mechanisms and to block relevant molecular pathways provides the most promising perspectives in the fight against gastrointestinal caner metastasis. And additionally, there are several interesting and enlightening hot points: the relationship between stromal microenvironment and metastatic destination; the role of cancer stem cells in metastasis; and how malignant cells get metastasis ability, inherent or acquired?

The second direction is to find out specific and sensible biomarkers of gastrointestinal cancer metastasis and to predict the potential metastasis, to facilitate early diagnosis, which is up to now mainly dependent on insensitive and costly radiographic examinations. And the third one is the optimization of comprehensive treatment including surgical intervention.

In this special issue, eight articles on gastrointestinal cancer metastasis are presented. One article discussed the advantages and disadvantages of repeated liver resection in patients with recurrent colorectal liver metastasis. One article reported that N-desulfated heparin is capable of suppressing the metastasis of gastric cancer through inhibiting tumor bFGF expression and tumor angiogenesis with no obvious anticoagulant activity. Yet another article is on the relevance between dietary salt intake and the risk of gastric cancer. The other five articles highlighted four kinds of potential biomarkers for gastrointestinal cancer screening and early diagnosis of metastasis, namely urine-free amino acid, ki-67 antigen, mir-21 and epidermal growth factor receptor (EGFR). J. Fan and his colleagues, through analyzing amino acids in urine samples of gastric cancer patients and healthy volunteers, figured out that urine-free amino acid profiling is of potential value for screening or diagnosing gastric cancer. D. Navarini and his colleagues, from Brazil, reviewed thirty-seven patients who underwent esophagectomy without presurgical chemotherapy or radiotherapy from 2000 to

2010, analyzed the level of EGFR expression and life span, and concluded that EGFR expression is related to higher TNM staging and shorter survival.

Despite the obvious advancements made in the research of gastrointestinal cancer metastasis these years, the outcomes are far from final satisfaction, and thus it is still a field that deserves and calls for intensive and thorough research in the future.

Acknowledgments

At last, please allow us to express our appreciation to all guest authors and researchers for their hard work and contribution to this special issue.

Jin-Lian Chen
Richard Ricachenevsky Gurski
Keiichi Takahashi
Roland Andersson

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Research Article

Urine Metabolite Profiling of Human Colorectal Cancer by Capillary Electrophoresis Mass Spectrometry Based on MRB

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Aim. The study was to investigate the metabolic profile of urine metabolites and to elucidate their clinical significance in patients with colorectal cancer. *Methods*. Colorectal cancers from early stage and advanced stage were used in this study. Urine samples of colorectal cancer patients and healthy adults were collected and subjected to capillary electrophoresis mass spectrometry based on moving reaction boundary analysis. The metabolic data were analyzed by SPSS 17.0 to find urinary biomarkers for colorectal cancer. *Results*. The results indicated that the urine metabolic profiling of colorectal cancer patients had significant changes compared with the normal controls, and there were also differences between early stage and advanced colorectal cancer patients. Compared with the control group, the levels of isoleucine, valine, arginine, lactate acid and leucine increased ($P < 0.05$), but those of histidine, methionine, serine, aspartic acid, citric acid, succinate, and malic acid decreased in urine samples from colorectal cancer ($P < 0.05$). Furthermore, the levels of isoleucine and valine were lower in urine of patients with advanced colorectal cancer than those in early stage colorectal cancer ($P < 0.05$). *Conclusion*. The technique of capillary electrophoresis mass spectrometry based on MRB could reveal the significant metabolic alterations during progression of colorectal cancer, and the method is feasible and may be useful for the early diagnosis of colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and a leading cause of cancer-associated death worldwide, especially in Europe and the United States [1–3]. Recently, the incidence rate of colorectal cancer has been increasing with lifestyle changes [4]. Though colorectal cancer is so common, we do not have effective treatment to cure it. Until now, the only curative treatment option for it is surgical resection [5]. However, the 5-year survival rates remain low, only 8% for stage IV patients, but are 93% for stage I patients [6]. So it is of significance to make a prediction about the oncogenesis and metastasis at the early stage of colorectal cancer in order to improve the prognosis. But we do not have effective measures to diagnose colorectal cancer at the time when the disease occurred as soon as possible for they have no specific clinical symptoms until in the late stage. To date, the most effective screening method for precancerous lesions and cancer morbidity in colon (e.g., aberrant crypt foci, polyps, and tumors) is colonoscopy [7]. However, the results of endoscopy are significantly affected by artificial factors (e.g., the experience of the gastrointestinal pathologist) [8, 9]; moreover, colonoscopy is invasive, and the procedure is unpleasant [2]. Although fecal occult blood testing (FOBT) and some certain tumor biomarkers, such as carcinoembryonic antigen (CEA), have been used in clinical dignosis, the sensitivity and specificity are relatively poor [10, 11]. Therefore, many researchers are trying to find novel biomarkers for the early detection of colorectal cancer.

In our study, we attempted to discover new biomarkers using a metabolomics approach for colorectal cancer. Metabolomics is the rapid development of new research areas following the genomics, transcriptome, and proteomics [12], the analytical method to study low molecular weight

compounds and of potential for development in system biology [4, 13], which can be used to analyze the changes in metabolite levels in biological samples [14]. Though functional genomics methods such as transcriptome and proteomics can simultaneously detect expression changes of a large number of genes or proteins under the influence of drugs, disease, environmental, or other factors, these changes could not establish direct relation with the changes of the biological functions. Metabolomics is the endpoint of the omics cascade in other words the last step in the cascade before the phenotype [4], and therefore it can establish a direct correlation for changes between metabolite levels and biological phenotype changes. At present, metabolomic methods include nuclear magnetic resonance spectroscopy (NMR), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis mass spectrometry (CE-MS) [15]. NMR requires a large number of samples as a result of the relatively poor sensitivity [12]; LC-MS needs derivation, which is a tedious complex process and is complicated to operate; GC-MS is of high sensitivity, but mainly for the analysis of volatile and semivolatile metabolites, and there are still some challenges for the nonvolatile metabolites [16]. Capillary electrophoresis mass spectrometry can overcome the shortcomings of the above methods, which not only has excellent resolution capability but also requires only trace amounts of sample. The main advantages of coupling capillary electrophoresis with mass spectrometry are the high resolution, good separation speed, sensitivity, and selectivity, which can detect almost any kind of charged compound [17]. Nevertheless, the detection sensitivity is limited due to the less injection of capillary electrophoresis. Our recent study has shown that moving reaction boundary (MRB) could stack analyte in capillary electrophoresis to improve the detection sensitivity [18]. In this study, we apply the technology of capillary electrophoresis mass spectrometry based on moving reaction boundary to achieve online enrichment and qualitative and quantitative detection of metabolites in the urine of patients with colorectal cancer.

2. Materials and Methods

2.1. Chemicals. Twenty kinds of amino acid solution and nine kinds of organic acid solution were purchased from Sigma (Sigma-Aldrich, Germany). HPLC-grade methanol (MeOH) was from Fisher Company. Formic acid (FA) (A.R) was supplied by Beijing Chemical Factory. Ammonium formate (A.R) was provided by Beijing Chemical Factory. Sodium hydroxide was purchased from West Long Chemical Co., Ltd., and Ammonia was from Hongdu Biochemistry Co., Ltd. (Jiangxi province, China). Standard solution was prepared with water from Milli-Q water purification system (Millipore).

2.2. Patients and Urine Samples. Urine samples were collected from 20 CRC patients (aged 37 to 87 years old) and 14 healthy volunteers (female/male, 6/8, aged 50 to 86 years old, and the median age was 68 years old) from Shanghai Sixth People's Hospital, Shanghai Jiaotong University (Shanghai, China). All the CRC patients were diagnosed by colonoscopy combined with pathological examination and stages according to the seventh edition of the International Union Against Cancer (UICC) TNM: stage I and II (early stage cancer), 8 patients (female/male, 5/3), aged 37 to 87 years (the median age was 77 years old); stage III and IV (advanced stage cancer), 12 patients (female/male, 5/7), aged 39 to 80 years (the median age was 69 years old). All subjects signed an informed consent under local research ethics committee approval. We collected the urine samples with centrifuge tubes of 5 mL temporarily early in the morning between 6.00 and 7.00 AM, centrifuged immediately; then the supernatants were transferred to frozen tubes and stored at -80°C until processing. All patients had not received chemotherapy or radiotherapy treatment before sample collection. Prior to CE-MS analysis, the urine samples were removed from the refrigerator to thaw at room temperature, transferred to centrifuge tubes of 1.5 mL, and centrifuged (12 000 rpm) for 10 min; subsequently the supernate was filtered through a 0.22 μm organic membrane. Mix the sample with sample buffer with the volume ratio of 1 : 1 before injection of samples into the capillary.

2.3. Instrumentation and Procedures. For CE-ESI-MS experiments, the HP^{3D}CE capillary electrophoresis instrument (Agilent, USA) was coupled to a 1100 series MSD TRAP (VL) mass spectrometer (Agilent, USA) applying a custom ESI sheath-flow interface with nebulizer gas [19]. Uncoated fused silica capillaries were from Ruifeng Chromatography Devices Co., Ltd. (Yongnian, Hebei, China) with a total length of 90 cm and an inner internal diameter of 50 μm. Formic acid (1.2 M, pH 1.8) was used as background electrolyte (BGE), while ammonium formate (60 mM, pH 10.2) was as the sample buffer. Sample injection was performed hydrodynamically for 80 s at 50 mbar. The volume of the sample plug injected corresponds to 61 nL. We rinsed the new dare fused-silica capillaries with 1 M sodium hydroxide for 30 min at 930 mbar, followed by deionized water for 20 min at 930 mbar, then 0.1 M sodium hydroxide for 20 min at 930 mbar, and followed by deionized water for 20 min at 930 mbar. At the start of the day, capillaries were flushed with deionized water for 15 min at 930 mbar and with 0.1 M sodium hydroxide for 15 min at 930 mbar and then with deionized water for 15 min at 930 mbar. Between the runs, we rinsed capillaries with deionized water for 2 min, 0.1 M sodium hydroxide for 3 min, deionized water for 2 min, and background buffer for 3 min in succession. During the rinsing step, the end plate voltage, the nebulizer capillary voltage, and capillary voltage were set to 0, which prevented these washing solutions from getting into the vacuum part of the mass. We set the capillary temperature at 25°C and performed the separation voltage at +30 kV. Spectra were collected with a time resolution of 0.5 s. Masses were obtained from 50–350 m/z. CE-MS coupling was realized by a coaxial sheath liquid interface (Agilent, USA) with methanol-water-formic acid (50 : 50 : 0.1, v/v/v) as sheath liquid. The following spray conditions were used: ESI in positive ionization mode voltage was 4.5 kV; sheath liquid

flow, 9 μL/min; dry gas temperature, 275°C; nitrogen flow, 10 L/min; nebulizer pressure, 0.5 bar.

2.4. Analytical Validation. Linearity of response for metabolites was evaluated by standard measurement of six concentrations ranging from 10 to 200 μM (10, 20, 50, 100, 150, and 200 μM). Linearity of response for the metabolites was also measured in the same concentration range in pooled human urine. The reliability of the analysis of CE/MS detection was verified by 5 endogenous urinary metabolites (L-arginine, L-histidine, L-aspartic acid, lactic acid, and citric acid) of representative. The standards of these metabolites were added into the blank urine in accordance with low (20 μM), middle (50 μM), and high (80 μM) concentration; each quality control (QC) sample was repeatedly measured three times continuously.

2.5. Data Analysis. CE-MS data were analyzed using principal component analysis (PCA) to differentiate the samples. Each sample was represented by a CE/MS TIC. The absolute peak area of each compound was calculated as the response after the peak areas of compound were integrated. t-test was applied to compare the differences between two groups. For three groups or more, one-way ANOVA test was performed. Data were expressed as mean ± SD. The differentially expressed compounds were considered statistically significant with P value < 0.05. Statistical analyses were carried out using SPSS 17.0 for windows.

3. Results

3.1. Detection of the Standards. The chromatogram of 18 kinds of amino acid standards was shown in Figure 1. From the figure, we can see that 16 kinds of amino acids were separated effectively under such conditions, and the peak times were concentrated in 10–17 minutes. Substances in the actual urine samples also showed good separation from the chromatogram in Figure 2.

3.2. Recoveries and Precision Results. The standards of metabolites (L-arginine, L-histidine, L-aspartic acid, lactic acid, and citric acid) were added into the blank urine in accordance with low (20 μM), middle (50 μM), and high (80 μM) concentration; each QC sample was repeatedly measured three times continuously. The result is showed in Table 1.

3.3. Evaluation of CE-ESI-MS Method for Metabolic Profiling. Recently, a CE-ESI-MS method has been developed for the highly efficient and sensitive analysis of metabolites (amino acids and organic acids) in human urine [19]. Here we demonstrate that MRB-CE-ESI-MS method can be used for the profiling of amino acids and organic acids. In CE, amino acids and organic acids (ionogenic compounds with electrophoretic properties) migrate toward the cathode along with the electroosmotic flow which is the MS in our setup. To analyze amino acids and organic acids simultaneously, 1.2 M formic acid with low pH was used to confer positive charges on these ionic compounds, making them amenable to MS analysis. Figure 1 shows chromatogram of 18 kinds of

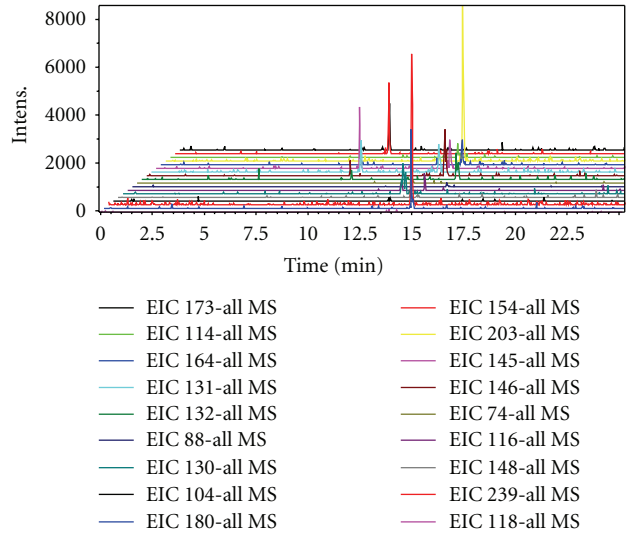

Figure 1: Total ion chromatogram of 18 kinds of amino acid standards.

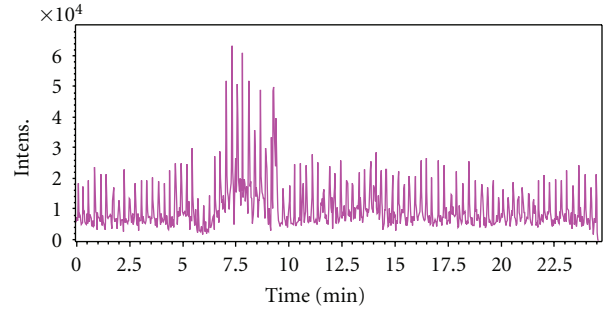

Figure 2: Total ion chromatogram of the urine of a patient with colorectal cancer.

amino acid standards; 16 amino acids were separated in less than 18 min. Figure 2 displays a total ion electropherogram of urine from a colorectal cancer patient obtained by CE-ESI-MS analysis; around 100 signals were detected in the sample. The analytical quality of the data obtained from the CE-ESI-MS analysis of QC samples was stated in Table 1. The five QC samples before and after addition of known analyte concentrations (20, 50, and 80 μM) were analyzed as shown in Table 1. Recoveries ranged from 71.83% to 88.70%, and the RSDs were 1.92%–6.70% for all analytes.

3.4. Metabolic Profiling and Multivariate Statistics. We used the CE-ESI-MS method for the metabolic profiling of urine samples from 20 patients with CRC and from 14 control subjects. All data was obtained by LC/MSD Trap Software Version 5.2 based on internal standard; most of the total ion chromatograms were identified as endogenous organic acids and amino acids involved in several metabolic pathways, such as glycolysis (lactic acid), serine metabolism (serine), and tricarboxylic acid (TCA) cycle (succinate, citric acid, and malic acid).

The CE-ESI-MS data about urine metabolites in CRC and normal control subjects were analyzed by t-test after

Table 1: Recoveries and precision of QC samples ($n = 3$).

Metabolite	Adding amount (μM)	Detection value (μM)	Average recovery (%)	RSD (%)
Arginine	20.0	16.0, 17.0, 18.0	85.00	5.88
	50.0	43.0, 44.0, 46.0	88.70	3.45
	80.0	68.0, 71.0, 73.0	83.33	2.29
Histidine	20.0	14.0, 15.0, 16.0	75.00	6.70
	50.0	43.0, 44.0, 45.0	88.00	2.27
	80.0	62.0, 65.0, 67.0	80.80	3.89
Aspartate	20.0	14.0, 15.0, 15.0	73.33	3.94
	50.0	39.0, 41.0, 42.0	81.33	3.76
	80.0	63.0, 65.0, 68.0	80.83	2.36
Lactic acid	20.0	15.5, 16.0, 17.3	81.33	5.71
	50.0	42.6, 44.2, 43.0	86.53	1.92
	80.0	68.5, 72.4, 70.2	87.96	2.78
Citric acid	20.0	13.6, 14.5, 15.0	71.83	4.94
	50.0	39.4, 41.6, 43.0	82.67	4.39
	80.0	68.3, 70.5, 74.0	88.67	4.05

normalization of data. The marker metabolites selected by t-test are displayed in Table 2. Among the metabolites, the lactic acid, arginine, isoleucine, leucine, and valine were significantly upregulated, while the citric acid, histidine, methionine, serine, aspartate, malic acid, and succinate were remarkably downregulated in CRC compared to the normal control subjects ($P < 0.01$). The main metabolic pathways related to CRC included glycolysis (lactic acid), serine metabolism (serine), tricarboxylic acid (TCA) cycle (succinate, citric acid, and malic acid). The levels of valine and isoleucine were lower in advanced stage colorectal cancer group than the early colorectal cancer group as shown in Table 3, $P < 0.01$.

A PCA model for colorectal cancer was constructed taking the marker metabolite areas as variables (lactic acid, arginine, isoleucine, leucine, valine, citric acid, histidine, methionine, serine, aspartate, malic acid, and succinate). The PCA scores plot illustrated that the CRC group and the normal control group were scattered into different regions (Figure 3(a)). ROC analysis was performed using the values of the first two principal components of the PCA model, which confirmed the reliability of the PCA. The specificity and sensitivity trade-offs were summarized for each variable with AUC. The AUC value of the PCA model is 1.00 (Figure 3(b)), which verified a good diagnostic value for colorectal cancer. Furthermore, another PCA model for advanced stage of CRC constructed by two metabolites could differentiate between the early stage group and the advanced group (Figure 4(a)). The PCA model was confirmed by the receiver operating characteristic (ROC) analysis with AUC = 0.906 (Figure 4(b)).

4. Discussion

Colorectal cancer is the second and fourth most cause of cancer-associated death in Europe and the USA, respectively [3]. In Singapore, colorectal cancer is the first most common cancer in males and the second in females; also CRC is the first most common cancer in Singapore when both genders are put together [20]. Recently, the number of colorectal cancer patients has been increasing because of the changes of lifestyle [4]. The main method to improve the prognosis of patients with colorectal cancer is early diagnosis and early treatment. Fecal occult blood test (FOBT) is an effective screening tool to colorectal cancer but the sensitivity is poor [4, 21]. Though the endoscopic examination of colon is the gold standard for diagnosis, it is invasive and unsuited to decrease the risk of morbidity and mortality [4]. The approaches used in colorectal cancer staging and prognosis assessment have many limitations [22]. Capillary electrophoresis mass spectrometry has been developed rapidly because of its many superiorities in detecting metabolites of urine and other body fluids, such as the high resolution power, good separation speed, and sensitivity and selectivity [17]. Despite this, the detection sensitivity is limited for the less injection of capillary electrophoresis. Moving reaction boundary (MRB) can stack analytes in capillary electrophoresis to improve the detection sensitivity [18]. In this method, a neutralization reaction is formed between the background buffer (1.2 M formic acid pH = 1.8) and the sample buffer (60 mM ammonium pH = 10.2), when an electrical field is imposed. The neutralization leads to the formation of a progressive low-conductivity zone in the original matrix plug, and then the low-conductivity zone further results in pH-mediated-induced field-amplified sample injection (FASI) stacking [23]. Moving reaction boundary was formed by the electromigration reaction which can be used to enrich the samples. The mechanism of preconcentration is as follows: the capillary is filled with background buffer of high conductivity, and the sample buffer is injected into the capillary to a certain length

Table 2: Different metabolites identified in normal control group and colorectal cancer group (mean ± SD).

Metabolites	Normal control group ($n = 14$)	Colorectal cancer group ($n = 20$)	P
Leucine	497444.00 ± 108999.397	1074016.00 ± 456521.184	0.000
Isoleucine	612788.64 ± 93219.817	1134260.20 ± 496322.174	0.000
Valine	538171.79 ± 100157.421	995357.85 ± 347273.028	0.000
Arginine	456031.93 ± 117404.005	1179011.50 ± 548364.933	0.000
Histidine	1168892.57 ± 317549.609	649361.35 ± 168817.630	0.000
Methionine	1261904.93 ± 468921.459	713061.95 ± 189689.901	0.000
Aspartate	1896296.43 ± 921667.854	681982.95 ± 122738.857	0.000
Serine	1023781.50 ± 203670.927	657044.45 ± 123487.631	0.000
Lactic acid	619754.64 ± 129620.274	1168716.15 ± 339239.261	0.000
Succinate	965363.21 ± 161307.936	611380.10 ± 144727.123	0.000
Citric acid	1298679.71 ± 610999.062	642247.10 ± 224070.728	0.002
Malic acid	1083173.43 ± 203204.078	613131.90 ± 164751.873	0.000

Table 3: Different metabolites identified in early stage group and advanced group (mean ± SD).

Metabolites	Early stage group ($n = 8$)	Advanced group ($n = 12$)	P
Valine	1315695.50 ± 337981.724	781799.42 ± 105973.802	0.003
Isoleucine	1584647.00 ± 463637.420	834002.33 ± 206929.304	0.000

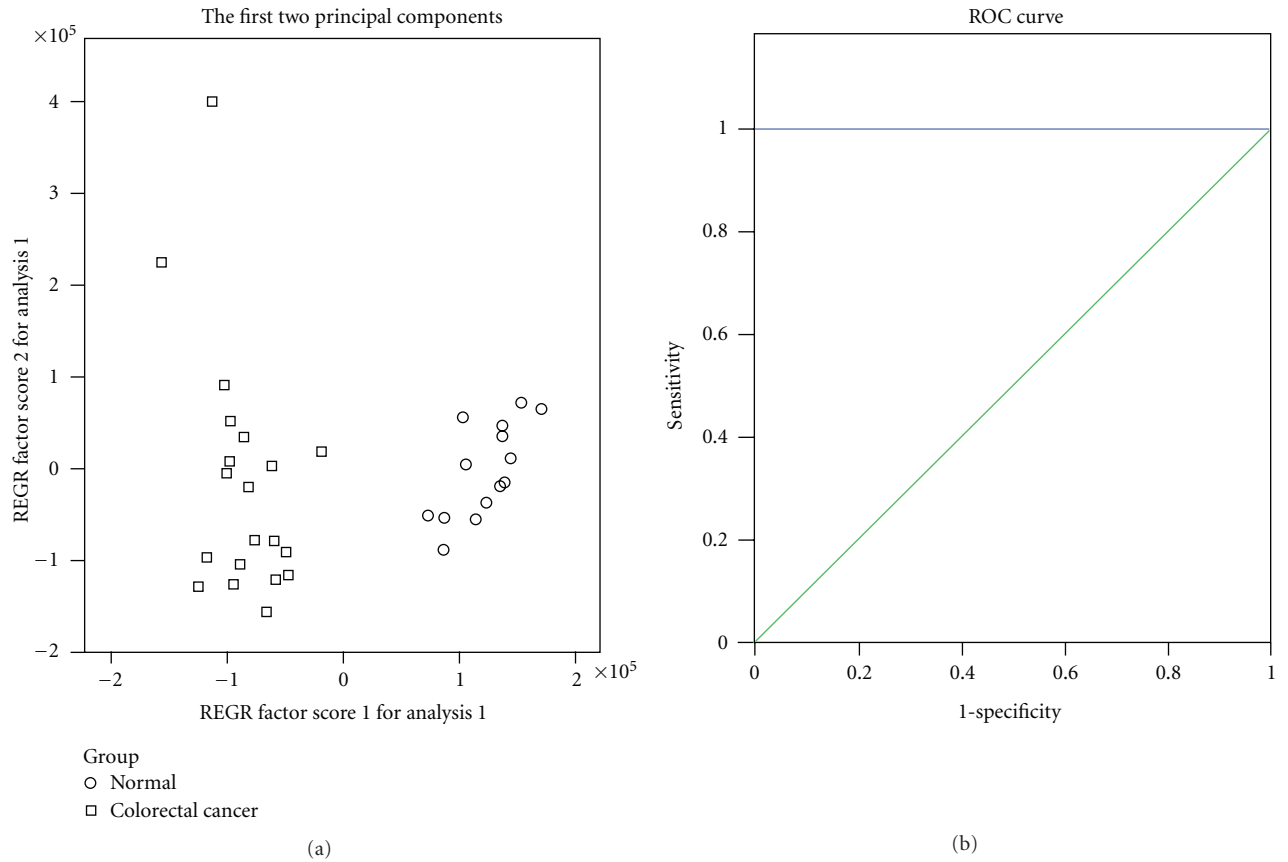

Figure 3: Principal component analysis model and receiver operating characteristic curve for colorectal cancer. (a) Principal component analysis (PCA) scores plot of colorectal cancer urine specimens from control specimens based on 12 marker metabolites. The PCA scores plot showed that different samples (normal group, colorectal cancer group including early stage group and advanced group) were scattered into different regions; (b) receiver operating characteristic (ROC) analysis was performed using the values determined by the first two principal components. Area under the curve (AUC) = 1.00.

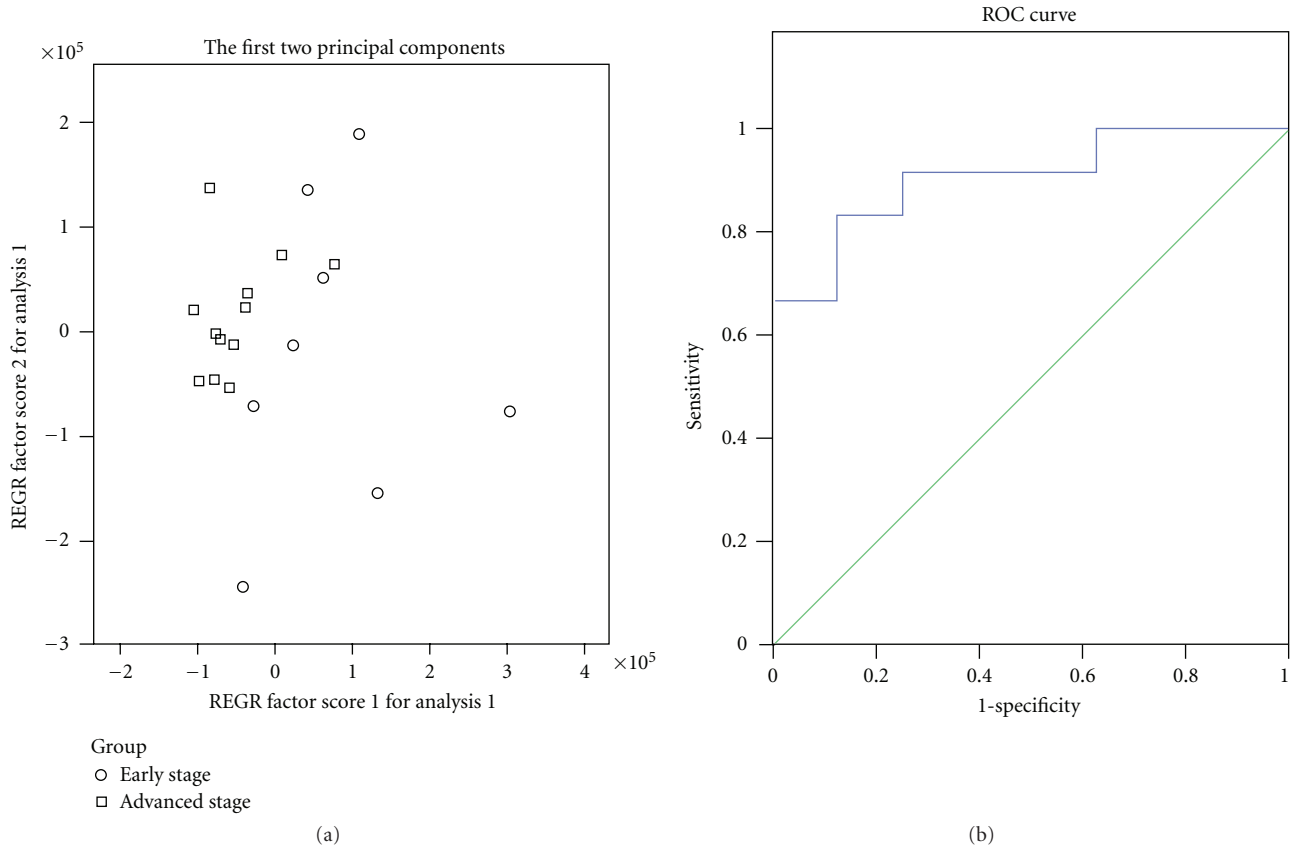

Figure 4: Principal component analysis model and receiver operating characteristic curve for advanced colorectal cancer. (a) Principal component analysis (PCA) scores plot of early stage group and advanced group based on 2 marker metabolites. The PCA scores plot showed that the samples from early stage group and advanced group were scattered into two different regions; (b) receiver operating characteristic (ROC) analysis was performed using the values determined by the first two principal components. Area under the curve (AUC) = 0.906.

subsequently. After the sample is injected completely, a high positive voltage is applied, the greater electric field causes the ions to migrate more rapidly across the sample zone. Once the ionic analytes arrive at the boundary between the sample zone and the background buffer, the electric field strength suddenly weakened and migration becomes slower, causing the sample analytes to be concentrated near the boundary. For the mobility of electroosmotic flow (EOF) is rapider than those of the charged analytes, all the charged analytes will finally move toward the mass spectrometry side, while the analytes in samples are separated by the CE mode [24]. To our knowledge, this is the first metabolomic investigation of urine with colorectal cancer using capillary electrophoresis mass spectrometry based on MRB.

In our study, twelve differential metabolites were found between colorectal cancer and normal control subjects, while two differential metabolites were identified between early stage and advanced colorectal cancer. PCA analysis showed there were significant differences among normal controls and colorectal cancer patients, including 8 patients with early colorectal cancer.

The lactic acid level was found to be higher in CRC urine. Lactic acid is the end product of glycolysis that increases rapidly during the occurrence and development of tumor. Cancer cells are in a high metabolic state, and the rate of aerobic glycolysis becomes higher, with more glucose converted into lactic acid even in the presence of oxygen, which is known as the "Warburg effect" [9, 20, 25]. The increased lactate level has been demonstrated in various tumors, including renal, head and neck, and gastric cancers [9, 26–28]. We found the lower levels of citric acid, malic acid, and succinate in the CRC urine specimens, which are most likely related to the deregulation of the tricarboxylic acid cycle and the increased demand for energy in tumors [20, 28]. The isoleucine is ketogenic amino acid which decomposed into acetyl-coenzyme A and succinatethe-coenzyme A, the important substances of the tricarboxylic acid cycle, which were greatly required during the gluconeogenesis [29], so the isoleucine level is higher in the cancer group compared to the normal group. The higher level of valine in CRC group than normal group is also related to the increased glycolysis in tumor cells [30]. Aspartic acid is one of the essential substances of the *in vivo* synthesis of nucleotides, which is needed in large amounts for the synthesis of nucleotides to promote tumor development when the tumor cells proliferate; therefore, the content of aspartic acid in the body is expressed at lower level than normal control group. Serine was decreased in colorectal cancer patients compared to the controls because serine takes part in glycolysis or tricarboxylic acid cycle to provide more energy for the progression

of tumor [31]. Arginine is known to be related to immune function [32]. It has been reported that arginine can inhibit chemical-induced colorectal cancer and reduce cell proliferation in colorectal adenoma patients [33, 34]. We found histidine was downregulated in CRC patients compared with normal control subjects. The mechanism may be the higher activity of histidine decarboxylase leading to the accelerated decarboxylation of histidine to histamine [2]. Methylation is one of the mechanisms for regulating cell growth and differentiation; S-adenosyl-L-methionine is a methyl donor which is generated by the conversion of methionine; therefore the level of methionine was down-regulated during the process of tumor occurrence and development [12].

In our study, we also found that the levels of valine and isoleucine were lower in the advanced colorectal cancer group compared with the early stage group. It could be explained by the hypothesis of Yamanaka and the workers [29]; obstacles produced by increasing muscle tissue protein improved the intrahepatic glucose gluconeogenesis and added the oxidation of BCAA in muscle, which is the main mechanism of the elevated levels of BCAA for patients with early colorectal cancer, but the further development of tumor can stop the increasing barriers, and therefore the BCAA levels may decline.

5. Conclusion

To the best our knowledge, this is the first report to evaluate the variations of urine metabolites among colorectal cancers using capillary electrophoresis mass spectrometry based on MRB. In our study, we filter out 12 marker metabolites between normal control subjects and colorectal patients and 2 marker amino acids between early stage and advanced colorectal cancers. The results suggest that the technique of capillary electrophoresis mass spectrometry based on MRB is feasible and may be useful for the diagnosis of colorectal cancer.

Conflict of Interests

The authors state no conflict of interests.

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Research Article

Association between Habitual Dietary Salt Intake and Risk of Gastric Cancer: A Systematic Review of Observational Studies

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Purpose. Systematic reviews of case-control and prospective studies showed a positive association between habitual salt intake and gastric cancer. Given new studies published thereafter, we carried out a meta-analysis to assess the association between dietary salt intake and gastric cancer. *Methods.* Case-control studies and cohort studies published between January 1992 and January 2012 on PubMed and Embase were searched. We quantified associations between salt intake and gastric cancer with meta-analysis. *Results.* Eleven studies (7 case controls and 4 cohorts) finally were included in the meta-analysis (total population: $n = 2076498$; events: $n = 12039$). The combined odds ratio showed significantly positive association between high salt intake and gastric cancer compared with low salt intake (OR = 2.05, 95% CI [1.60, 2.62]; $P < 0.00001$). In subgroup meta-analysis, findings were slightly different when analyses were restricted to salty food intake (OR = 2.41, 95% CI [2.08, 2.78]; $P < 0.00001$) as well as in Asia (OR = 1.27 95% CI [1.22, 1.32]; $P < 0.00001$). There was no evidence that sample size, exposure assessment substantially influenced the estimate of effects. *Conclusions.* The systemic review supports the hypothesis that dietary salt intake is positively associated with the risk of gastric cancer.

1. Introduction

Dietary factors are important environmental risk determinants for cancer development. The role of dietary factors in gastric cancer was studied in the last 40 years and received particularly attention recently. It is difficult to establish a causal relationship between salt intake and gastric cancer due to methodological limitations among which is the valid measurement of accurate salt intake. Therefore, the conclusion is still unclear. In 2007, the Second Expert Report from the World Cancer Research Fund [1] supported the view that salt intake is significantly related to gastric cancer. From then on, two meta-analyses have been published addressing the association between salt intake and gastrointestinal metaplasia or gastric cancer. The study [2] on salt intake and gastrointestinal metaplasia revealed a positive trend but no statistical significance was observed. Another meta-analysis [3] included 7 prospective studies in total, and four out of seven were carried out in Japan. Although it demonstrated a positive association between salt consumption and incidence rate of gastric cancer, the limitation in geographic location prevents its generalization. Although prospective studies have more power in controlling confounders than case-control studies, the tendency of using baseline salt intake to represent the subsequent salt consumption causes inaccuracy in actual salt intake. Therefore, we carried out this systematic review to assess the relationship between habitual dietary salt intake and risk of gastric cancer.

2. Materials and Methods

2.1. Data Sources and Searches. This paper was planned, conducted, and reported according to the PRISMA statement [4]. We performed a systematic search for publications using MEDLINE and Embase databases (from 1992 to 2012). The following keywords were used in searching: "salt or sodium or salty or sodium chloride" and "gastric cancer or stomach cancer." Moreover, we searched for the keywords in titles,

abstracts and performed a manual search of references cited in the selected articles and published reviews.

2.2. Eligibility Criteria. Citations selected from the initial search were subsequently screened for eligibility. The list of references was independently screened by two reviewers. Cohort and case-control studies were included in the systematic review when all the following criteria were met: (1) original research addressing the association between the consumption of salt or salted foods and the occurrence of gastric cancer in humans; (2) prospective design; (3) adult population; (4) exposure defined as salt or salted foods by the authors of each study or including foods with high contents of salt as defined in the latest report of the World Cancer Research Fund and the American Institute for Cancer Research (processed foods such as processed meat; salty foods such as potato crisps or chips, salted nuts, and salty snack foods; salted foods such as bacon, sausages, and ham; salt-preserved foods such as salted meat, fish, vegetables, and fruits); (5) diagnosis of gastric cancer determined prospectively as outcome (gastric cancer incidence and/or mortality rate); (6) indication of the number of participants exposed and the rate or number of events in different categories of salt/sodium intake; (7) articles written in English.

2.3. Data Extraction and Quality Assessment. We designed a data collection form before selecting eligible studies. The following data were extracted independently by two authors using a unified data form, the first author's full name, year of publication, country, gender, age, range of followup, study population, the events and mortality of gastric cancer, types of estimate of habitual sodium intake, and factors controlled and matched or adjusted variables in the design or data analysis. The results of the two authors were compared, and a consensus result would be achieved if there were any disagreement.

The Newcastle-Ottawa Scale [5] was used by two authors independently to evaluate the quality of the included studies. The Coding Manual for Case-Control Studies (an 8-stars system) was used to assess the quality of case-control studies, in which a study was judged on 3 broad perspectives as follows: (a) the selections of both study groups, (b) the comparability of study groups, and (c) exposure levels of study groups. The Coding Manual for Cohort Studies (a 9-star system) was used to assess the quality of cohort studies, as well as in case-control study. Each cohort study was judged on 3 broad perspectives as follows: (a) the selections of both study groups, (b) the comparability of study groups, and (c) outcome of the two study groups. The results of the two authors were compared, and a consensus result would be received if there was any disagreement.

2.4. Statistical Methods. We evaluated the differences between low salt consumption and high salt consumption on the occurrence of gastric cancer. The original data from the studies was used to calculate the summary OR with its 95% confidence intervals (CIs) in all analyses. A random-effects model was used to account for between-study heterogeneity and publication bias.

The method of Mantel-Haenszel test [6] was used to assess the pooled OR and corresponding 95% confidence intervals (CIs) across studies. Forest plots were used to visually assess the results of Mantel-Haenszel test.

To assess the heterogeneity of ORs across studies, the Cochran Q statistic [7] (significance level of $P < 0.10$) and the I^2 statistic [8] (which quantifies the percentage of variation attributable to heterogeneity) were calculated. Subgroup analyses were carried out by region and food items to assess the relationship between salt intake and gastric cancer. The Begg test [9] and funnel plot [10] were used to assess the possibility of publication bias.

3. Results

3.1. Literature Search. Our search strategy yielded 1580 articles: 810 from Embase and 770 from Pubmed. A flow diagram that detailed the process is presented in Figure 1. The main details of the flow diagraph are as follows: after the first step, only 492 articles with full-text access are reserved. There were 159 reviews, 14 letters or editorial, and 50 duplicated articles among the 492 articles. 242 articles were excluded after we had reviewed titles and abstracts, and only 27 articles are reserved. 16 articles were excluded after reviewing the full text, because there were no original data. Finally, 11 studies (7 case-control studies [11–17] and 4 cohort studies [18–21]) were included in the meta-analysis.

3.2. Characteristics of the Included Studies. The characteristics of the studies included in our meta-analysis are showed in Table 1 [11–21]. The total number of participants was 2076498 [11–20, 20, 21]. The study design types were as follows: case-control study ($n = 7$ [11–17, 21]) and cohort study ($n = 4$ [18–21]). Studies were conducted in Japan ($n = 3$ [19–21]), Korea ($n = 2$ [16, 18]), China ($n = 1$ [17]), Spain ($n = 1$ [14]), Portugal ($n = 1$ [11]), Colombia ($n = 1$ [12]), Iran ($n = 1$ [13]), and Mexico ($n = 1$ [15]). Only 2 [18, 20] of the final 11 studies reported women and men independently. Total salt intake was used to assess sodium intake in 4 studies [11–13, 20]. Salted food was used in 6 studies [14–17, 20, 21]. Salt preference was used in the last 1 study [18].

The study quality scores were listed in Table 2: 6 studies got a score of 8 (4 case-control studies and 2 cohort studies), 5 studies got 7 points (3 case-control studies and 2 cohort studies).

3.3. The Risk of Gastric Cancer of High Salt Intake and Low Salt Intake. The multivariable-adjusted ORs for each study and combination of all studies for the high versus low categories of salt intake are shown in Table 3. The summary OR of all studies, using a random-effects model, showed that the high salt intake was significantly associated with a 105% greater risk of gastric cancer compared with low salt intake (OR = 2.05 95% CI [1.60, 2.62]; $Z = 154.7$; $P < 0.00001$). However, there was statistically significant heterogeneity across

Table 1: The characteristics of the studies included in the meta-analysis.

First author	Publication year	Location	Study period	Sex	Age (years)	Study population (n)	Gastric cancer (n)		Type of estimate of habitual sodium intake	Factors controlled for in multivariate analysis
							Events	Mortality		
Peleteiro [11]	2011	Portugal	2001–2006	F/M	18–92	1071	422	~	Total salt intake	Age, gender, education, smoking, *H. pylori* infection, and total energy intake
Campos [12]	2006	Colombia	2000–2002	F/M	49–75	647	216	30	Total salt intake	Age, gender, and hospital
Pourfarzi [13]	2009	Iran	2003–2005	F/M	65.46 ± (11.5)	611	217	~	Total salt intake	Age, gender, residence, education, and family history on cancer
Kim [18]	2010	Korea	1996–2003	F/M	30–80	2248129	12393	~	Salt preference	Age, sex, BMI, smoking habits, alcohol consumption, physical activity, and family history of cancer
Ramón et al. [14]	2002	Spain	1986–1989	F/M	32–80	305	165	12	Smoked and pickled foods, salt	Age, gender
Shikata [19]	2006	Japan	1998–2002	F/M	>40	2467	93		Total salt intake	Age, sex, *Helicobacter pylori* infection, atrophic gastritis, medical history of peptic ulcer, family history of cancer, body mass index, diabetes mellitus, total vitamin B2, vitamin C and dietary fiber intake, smoking habits and dietary factors intake of total energy, total protein, carbohydrate, vitamin B1, cholesterol, physical activity, and alcohol
Ngoan [20]	2002	Japan	1986–2001	F/M	>15	13250	116	116	Pickled food	Age, sex, smoking, and other dietary factors

Table 1: Continued.

First author	Publication year	Location	Study period	Sex	Age (years)	Study population (n)	Gastric cancer (n)		Type of estimate of habitual sodium intake	Factors controlled for in multivariate analysis
							Events	Mortality		
Ward [15]	1999	Mexico	1989–1990	F/M	~	972	220	~	Salty snacks	Age, gender, and total calories
Tsugane [21]	2004	Japan	1990–2001	F/M	40–59	39065	486	361	Salted food consumption (miso soup, pickled vegetables, salted fish roe, salted fish preserves, and dried or salted fish)	Age, gender, cigarette smoking, fruit and vegetable intake, drinking history, and personal medical history
Lee [16]	2003	Korea	1999.3–1999.9	F/M	~	268	69	~	Salt-fermented fish	Age, sex, education, family history of gastric cancer, smoking, alcohol drinking, and *H. pylori* infection
Yang [17]	2011	China	2006–2010	F/M	40–75	900	300		Salted food (meat and fishes, pickled vegetable)	Age, sex, smoking, drinking, fresh fruit, and fresh vegetables

Table 2: The study quality scores of the studies included in meta-analysis.

First author, year of publication (reference)	Objects selection				Comparability	Exposure/result cases and controls			Total quality scores
	Adequate definition/exposed cohort representat-iveness	Representativeness of cases/nonexposed cohort	Controls selection/exposure ascertainment	Controls definition/outcome not present		Exposure ascertainment/outcome assessment	ascertainment method/followup length	Nonresponse rate/adequate followup	
Campos, 2006 [12]	⋆	⋆	0	⋆	⋆⋆	⋆	⋆	0	7
Tsugane, 2004 [21]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Peleteiro, 2011 [11]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Pourfarzi, 2009 [13]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Yang, 2011 [17]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Shikata, 2006 [19]	0	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	7
Ward, 1999 [15]	0	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	7
Ngoan, 2002 [20]	0	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	7
Ramón et al. , 1993 [14]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Lee, 2003 [16]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8
Kim, 2010 [18]	⋆	⋆	⋆	⋆	⋆⋆	⋆	⋆	0	8

Figure 1: Process of study selection for salt intake and risk of gastric cancer.

the studies ($P < 0.01$; $I^2 = 92\%$), regarding the methods were used to evaluate the exposure, the food items evaluated, the consumption categories considered for analysis, and the degree of adjustment for possible confounders. Subgroup analysis was used for categorical variables.

A trend toward a direct association between salt intake and gastric cancer risk was detected in all 11 individual studies that were included in the meta-analysis and statistically significant in 9 of them.

3.4. Subgroup Analysis. Stratifying by geographic region, the pooled ORs of gastric cancer for the high versus low categories of salt intake were 1.15 (95% CI, [0.88, 1.52]) for studies conducted in Europe, there was no statistically significant heterogeneity among studies of salt intake in Europe ($P = 0.19$ and $I^2 = 41\%$) (Table 4); and 1.27 (95% CI, [1.22, 1.32]) for studies conducted in Asia with stratification according to geographic region, and there was statistically significant heterogeneity among studies of salt intake in Asia ($P < 0.00001$ and $I^2 = 95\%$) (Table 5). So separate analysis of the studies reporting further analyses was carried out to check for potential sources of heterogeneity that might explain the association between dietary salt intake and gastric cancer events in Japan. The OR was 2.61 (95% CI, [2.02, 3.38]) for studies conducted in Japan (Table 6).

We also conducted analyses that were stratified according to the food items, using studies that reported results on gastric cancer in relation to food items. The OR for salt was 1.20 (95% CI, [1.15, 1.26]), and there was statistically significant heterogeneity among these studies ($P = 0.03$ and $I^2 = 67\%$) (Table 7). The OR for salty food was 2.41 (95% CI, [2.08, 2.78]), statistically significant heterogeneity was also found among these studies ($P < 0.000\,01$ and $I^2 = 89\%$) (Table 8). The results showed that different sources of salt intake (salt or food items) had different risks on gastric cancer.

4. Discussion

Diet is considered to be associated with carcinogenesis. In this meta-analysis, we attempted to collect the evidence to identify the relationship between dietary salt intake and gastric cancer. Findings from the current study suggested that, compared with low salt intake, high salt intake showed significantly positive association with gastric cancer (overall OR = 2.05, 95% CI [1.60, 2.62]; $P < 0.00001$). However, there was a significant heterogeneity among the included studies. In subgroup analysis by category of salt intake, geographical regions, and sex, however, the significantly positive association was not changed.

Although we observed a positive association, there were many methodological limitations in human studies which prevent valid measurements used to assess salt consumption effectively [24]. For example, there are greater recall and selection biases in case-control studies because of their retrospective nature. In these studies, gastric cancer patients were more likely to change their dietary behavior as well as salty foods for their health. Then their earlier long-term dietary habit may have been strongly influenced by the recent diet. Because of different methods used to assess and report

Table 3: High versus low categories of salt and gastric cancer.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Peleteiro et al. 2011 [11]	144	364	132	346	2.0%	1.06 [0.78, 1.44]	
Pourfarzi et al. 2009 [13]	121	213	95	394	0.7%	4.14 [2.90, 5.91]	
Campos et al. 2006 [12]	19	30	197	617	0.2%	3.68 [1.72, 7.89]	
Kim et al. (men) 2010 [18]	2261	302734	5874	890825	73.9%	1.13 [1.08. 1.19]	
Kim et al. (women) 2010 [22]	518	136400	1796	560116	17.5%	1.19 [1.07, 1.31]	
Jose M. Ramón et al. 1993 [14]	27	63	33	108	0.3%	1.70 [0.89, 3.25]	
Shikata et al. 2006 [19]	30	564	12	663	0.3%	3.05 [1.55, 6.01]	
Ngoan et al. (men) 2002 [20]	7	341	27	2039	0.2%	1.56 [0.67, 3.62]	
Ngoan et al. (women) 2002 [20]	1	316	8	2876	0.0%	1.14 [0.14, 9.13]	
Ward and López-Carrillo 1999 [15]	105	355	75	435	1.2%	2.02 [1.44,2.83]	
Tsugane et al. 2004 [21]	130	89048	58	86483	1.5%	2.18 [1.60, 2.97]	
Lee et al. 2003 [16]	47	69	22	199	0.1%	17.19 [8.77, 33.68]	
Yang et al. 2011 [17]	180	482	120	418	2.0%	1.48 [1.12, 1.96]	
Total (95% CI)		**530979**		**1545519**	**100.0%**	**1.22 [1.17, 1.27]**	
Total events	3590		8449				
Heterogeneity: $Chi^2 = 154.77$, df = 12 ($P < 0.00001$); $I^2 = 92\%$							
Test for overall effect: $Z = 9.64$ ($P < 0.00001$)							

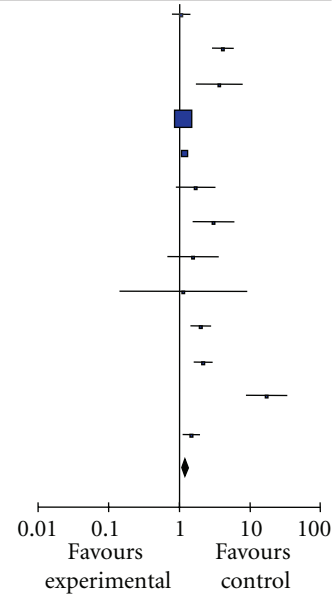


Table 4: High versus low categories of salt and gastric cancer in Europe.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Peleteiro et al. 2011 [23]	144	364	132	346	85.5%	1.06 [0.78, 1.44]	
Jose M. Ramón et al. [14]	27	63	33	108	14.5%	1.70 [0.89, 3.25]	
Total (95% CI)		**427**		**454**	**100.0%**	**1.15 [0.88, 1.52]**	
Total events	171		165				
Heterogeneity: $Chi^2 = 1.70$, df = 1 ($P = 0.19$); $I^2 = 41\%$							
Test for overall effect: $Z = 1.03$ ($P = 0.30$)							

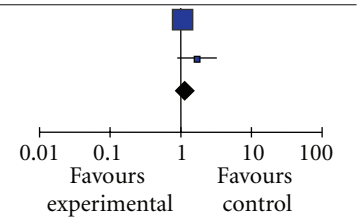


Table 5: High versus low categories of salt and gastric cancer in Asia.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Pourfarzi et al. 2009 [13]	121	213	95	394	0.8%	4.14 [2.90, 5.91]	
Campos et al. 2006 [12]	19	30	197	617	0.2%	3.68 [1.72, 7.89]	
Kim et al. 2010 [18]	2779	439134	7670	1450941	94.7%	1.20 [1.15, 1.25]	
Shikata et al. 2006 [19]	30	564	12	663	0.3%	3.05 [1.55, 6.01]	
Ngoan et al. 2002 [20]	8	657	35	4915	0.2%	1.72 [0.79, 3.72]	
Tsugane et al. 2004 [21]	130	89048	58	86483	1.6%	2.18 [1.60, 2.97]	
Lee et al. 2003 [16]	47	69	22	199	0.1%	17.19 [8.77, 33.68]	
Yang et al. 2011 [17]	180	482	120	418	2.2%	1.48 [1.12, 1.96]	
Total (95% CI)		**530197**		**1544630**	**100.0%**	**1.27 [1.22, 1.32]**	
Total events	3314		8209				
Heterogeneity: $Chi^2 = 133.98$, df = 7 ($P < 0.00001$); $I^2 = 95\%$							
Test for overall effect: $Z = 11.22$ ($P < 0.00001$)							

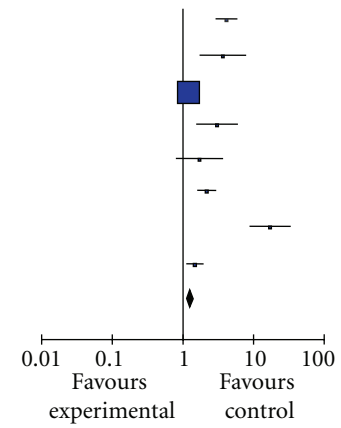


Table 6: High versus low categories of salt and gastric cancer in Japan.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Campos et al. 2006 [12]	19	30	197	617	8.7%	3.68 [1.72, 7.89]	
Shikata et al. 2006 [19]	30	564	12	663	13.6%	3.05 [1.55, 6.01]	
Ngoan et al. 2002 [20]	8	65	35	4915	1.0%	19.57 [8.69, 44.04]	
Tsugane et al. 2004 [21]	130	89048	58	86483	76.6%	2.18 [1.60, 2.97]	
Total (95% CI)		**89707**		**92678**	**100.0%**	**2.61 [2.02, 3.38]**	
Total events	187		302				
Heterogeneity: $Chi^2 = 25.98$, df = 3 ($P < 0.00001$); $I^2 =$ 88%							
Test for overall effect: $Z = 7.29$ ($P < 0.00001$)							

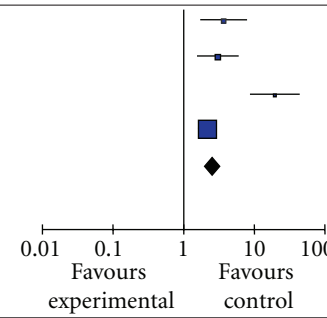


Table 7: High versus low categories of salt and gastric cancer through salt.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Peleteiro et al. 2011 [11]	144	364	132	346	2.2%	1.06 [0.78, 1.44]	
Kim et al. 2010 [18]	2779	439134	7670	1450841	97.1%	1.20 [1.15, 1.25]	
Jose M. Ramón et al. [14]	27	63	33	108	0.4%	1.70 [0.89, 3.25]	
Shikata et al. 2006 [19]	30	564	12	663	0.3%	3.05 [1.55, 6.01]	
Total (95% CI)		**440125**		**1451958**	**100.0%**	**1.20 [1.15, 1.26]**	
Total events	2980		7847				
Heterogeneity: $Chi^2 = 9.01$, df = 3 ($P = 0.03$); $I^2 = 67\%$							
Test for overall effect: $Z = 8.43$ ($P < 0.00001$)							

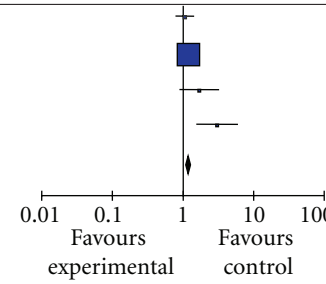
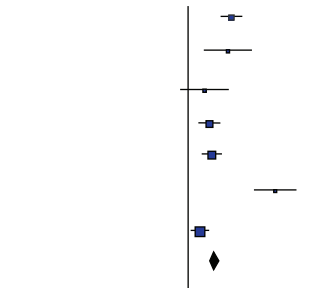


Table 8: High versus low categories of salt and gastric cancer through salty food.

Study or subgroup	High salt		Low salt			Odds ratio	Odds ratio
	Events	Total	Events	Total	Weight	M-H, fixed, 95% CI	M-H, fixed, 95% CI
Pourfarzi et al. 2009 [13]	121	213	95	394	12.3%	4.14 [2.90, 5.91]	
Campos et al. 2006 [12]	19	30	197	617	2.9%	3.68 [1.72, 7.89]	
Ngoan et al. (men) 2002 [20]	8	657	35	4915	3.5%	1.72 [0.79, 3.72]	
Mary H. Ward 1999	105	355	75	435	20.3%	2.02 [1.44, 2.83]	
Tsugane et al. 2004 [21]	130	89048	58	86483	25.1%	2.18 [1.60, 2.97]	
Lee et al. 2003 [16]	47	69	22	199	1.5%	17.19 [8.77, 33.68]	
Yang et al. 2011 [17]	180	482	120	418	34.4%	1.48 [1.12, 1.96]	
Total (95% CI)		**90854**		**93461**	**100.0%**	**2.41 [2.08, 2.78]**	
Total events	610		602				
Heterogeneity: $Chi^2 = 56.59$, df = 6 ($P < 0.00001$); $I^2 = 89\%$							
Test for overall effect: $Z = 11.75$; ($P < 0.00001$)							



salt consumption across studies, we could not evaluate a dose-response relation between salt consumption and gastric cancer. We cannot be able to exclude the other confounding factors, such as mutagens in the salty foods or processed food.

The World Cancer Research Fund and the American Institute for Cancer Research published in 2007 a large systematic review and meta-analysis, which concerned the effect of salt in the development of gastric cancer [1]. A 17-article systematic review and meta-analyses on addressing the association between dietary salt exposure and gastric intestinal metaplasia received a positive association [2]. A meta-analysis (7 articles included) to assess the association between habitual salt intake and risk of gastric cancer

in prospective studies also got a positive association [3]. However, there was a significant heterogeneity among the included studies in the three articles.

4.1. Potential Mechanisms. Several mechanisms which suggested that salt intake may increase gastric cancer risk have been postulated although there has been no consistent conclusion.

4.1.1. The Destruction of the Mucosal Barrier. Intragastric high salt concentration destroys the mucosal barrier, through the increase of surface mucous cell mucin and decrease of gland mucous cell mucin [25], leading to inflammation and damage such as diffuse erosion and degeneration [21], produces atrophic gastritis and decreases the acidity of the stomach. It creates a condition favoring *H. pylori* infection [12].

4.1.2. Intestinal Metaplasia. Intestinal metaplasia is also an important risk factor of gastric cancer. Mucosal damage in the stomach increases the rate of mitosis, leading to excessive cell replication [22] and hyperplasia of the gastric pit epithelium with increased potential for mutations [23]. Intestinal metaplasia characterized by the presence of caliciform cells in glands and in foveolar gastric mucosa was detected near regenerative hyperplasia foci high NaCl diets animals [26]. High salt intake will increase concentration of NaCl in the stomach; then it may accelerate the procedure of intestinal metaplasia and increase the risk of gastric cancer in the future.

4.1.3. Hypergastrinemia. Gastrin itself may mediate epithelial cell growth in *H. pylori*-colonized mucosa [27] and induce hypergastrinemia [28]. Chronic hypergastrinemia can synergize with *Helicobacter* infection and lead to eventual parietal cell loss and progression to gastric cancer [29].

4.1.4. H. pylori. *H. pylori* is one of the important recognized risk factors of gastric cancer. The damage caused by salt may also increase gastric *H. pylori* colonization. *H. pylori* responds to changes in the concentration of NaCl in its environment in such a way that growth, cell morphology, survival, and virulence factor expression are all altered by increased salt concentration [30]. Elevated salt concentrations result in alterations in expression of the virulence factor CagA in *H. pylori* strain 26695 and enhance the ability of CagA to translocate into gastric epithelial cells and enhance the ability of *H. pylori* to alter gastric epithelial cell function [31].

4.1.5. Endogenous Mutations. Salt may also directly damage gastric mucus, improve inflammatory responses of the gastric epithelium [32], which may increase epithelial cell proliferation as part of the repair process, potentiate the action of carcinogens [33], and increase the probability of endogenous mutations [34].

4.1.6. Exposure to Carcinogens. High dietary salt intake damages the stomach mucosa that protects the stomach and increases the susceptibility of the mucosal cells to carcinogens from foods, such as N-nitroso compounds. And its repair is associated with inflammatory changes [35] and leads to cell death [36]. But the studies included in the current meta-analysis did not report the potential carcinogens of the salty foods or processed food. Future observational studies should pay more attention in this area.

4.2. Limitations. Despite these advantages, the current meta-analysis, however, had limitations.

First, the majority of included studies used questionnaire to assess habitual salt intake which had limited value [37]. Only few studies used 24 h urinary sodium excretion as indictor of salt consumption which is recommended by the World Cancer Research Fund as the best measurement of salt intake [38]. The information derived from the questionnaire is subjective, qualitative and had not covered all the sources of sodium intake [39]. In most studies, the consumption of salted food which is high in salt and nitrites as well was recorded as a source of sodium intake. It is well known that nitrite is a mutagen that is closely related to gastric carcinogenesis [40, 41]. These methodological limitations compromised the association between salt intake and gastric cancer, either toward exaggeration or underestimation of risk estimates.

Second, due to the huge heterogeneity of the related data presented in the studies, the number of studies involved in the meta-analysis was relatively small. Therefore, subgroup analyses were difficult to perform.

Third, the current meta-analysis is unable to rule out the possible influence of confounding factors on the revealed association. Some confounders were inherent in the included studies. Although each study recruited some known risk factors for adjustment for gastric cancer, these covariates were not consistent and unknown confounders such as mutagens in salted foods cannot be excluded as a potential explanation for the observed findings.

Fourth, the cutoff values corresponding to the low and high categories for salt intake varied widely among the studies, which might also affect the obtained results.

In conclusion, the overall current literature on dietary salt intake and the risk of gastric cancer suggested significantly positive association. Due to the nature of the association, more well-designed prospective studies that use unified measures of dietary salt intake are needed to fully characterize such an association, and it is impossible to perform a large randomized, controlled clinical trial to clarify the cause-effect relationship. Therefore, future observational study with recommended salt assessment method and maximized exclusion of confounders from salted foods is necessary.

Conflict of Interests

The authors declare that there was no conflict of interests.

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Research Article

Repeated Liver Resection for Colorectal Liver Metastases: A Comparison with Primary Liver Resections concerning Perioperative and Long-Term Outcome

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Introduction. 60% of patients operated for colorectal liver metastases (CRLM) will develop recurrent disease and some may be candidates for a repeated liver resection. The study aimed to evaluate differences in intraoperative blood loss and complications comparing the primary and the repeated liver resection for metastases of colorectal cancer (CRC), as well as to evaluate differences in long-time follow-up. *Method*. 32 patients underwent 34 repeated liver resections due to recurrence of CRLM an studied retrospectively to identify potential differences between the primary and the repeat resections. *Results*. There was no 30-day postoperative mortality or postoperative hospital deaths. The median blood loss at repeat resection (1850 mL) was significantly ($P = 0.014$) higher as compared to the primary liver resection (1000 mL). This did not have any effect on the rate of complications, even though increased bleeding in itself was a risk factor for complications. There were no differences in survival at long-term follow-up. *Discussion*. A repeated liver resection for CRLM was associated with an increased intraoperative bleeding as compared to the first resection. Possible explanations include presence of adhesions, deranged vascular anatomy, more complicated operations and the effects on the liver by chemotherapy following the first liver resection. 30 out of 32 patients had only one reresection of the liver.

1. Introduction

The second most common cause of cancer-related deaths worldwide is colorectal cancer (CRC), ranking second in Europe and third in the USA [1].

Surgical resection, if possible, is the standard treatment for patients with a localized tumour, but about 50–75% of patients with CRC will develop colorectal liver metastases (CRLM) [2–7]. Even in patients with advanced CRC disease, the liver may be the sole organ with metastases, which is the case in about 30% of the patients [8, 9]. In these cases, a resection of the CRLM may be potentially curative.

Without treatment, patients diagnosed with CRLM have a median survival time around 8–15 months with a 5-year survival rate of 5% [10–13]. With powerful chemotherapy, the median survival time increases up to as much as two years [14].

1.1. Objective. The aim was to study the difference between the primary and repeated liver resection for CRLM, especially as comes long-term survival, intraoperative bleeding, and rate of complications.

1.2. General Treatment of Colorectal Liver Metastases. Liver resection is, when possible, considered the standard treatment of choice for CRLM. Other types of treatment are methods for local tumour destruction, including radiofrequency ablation. These treatments are most often used in patients with nonresectable liver metastases, but may be used as a part of neoadjuvant and adjuvant treatment, and together with surgical resection in order to improve the results following surgery [15].

The main issue in adjuvant treatment following liver resection concerns chemotherapy. Chemotherapy is otherwise generally palliative in the treatment of metastases

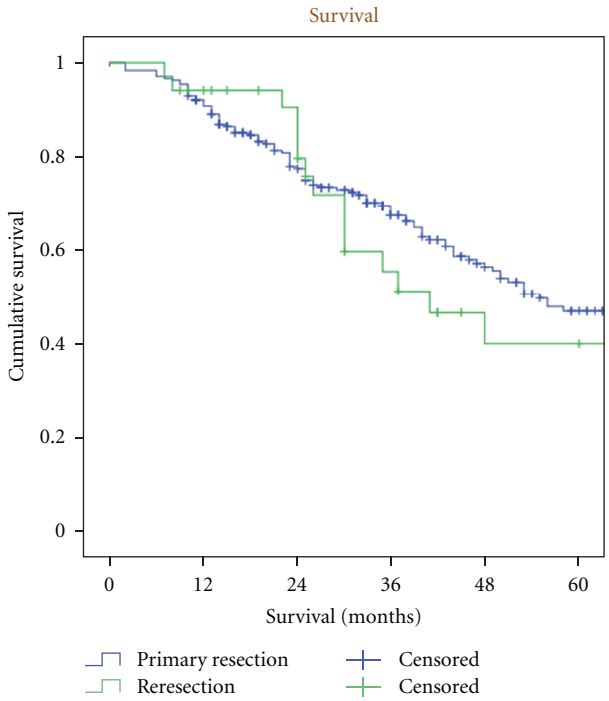

Figure 1: Kaplan-Meier estimate on survival following primary liver resection for colorectal liver metastases and reresection of the liver (n.s.).

of CRC, but may also prolong the survival time [14]. However, chemotherapy can also be used both as neoadjuvant treatment for downsizing the liver metastases, with our without PVE (portal vein embolization) or other local treatment, making otherwise unresectable tumours surgically respectable. Chemotherapy may also be given as adjuvant treatment following liver resection in order to at least lengthen the period until potential tumor recurrence after the initial liver resection for CRLM happens [16, 17].

1.3. Resection of Colorectal Liver Metastases. The 5-year survival rate after resection of CRLM varies broadly in different reports, ranging between 15–50%, but in more recent studies the range is usually 40–50% [2, 18–21].

20–30% of patients with CRLM directly fulfil criteria which make them suitable for liver resections. The criteria for operating CRLM have changed over time, from looking on what can be removed, to also include optimization of what will remain of the liver. Overall, with all novel treatment options, an increased number of patients with CRLM can be offered the surgical option [2, 22–24].

1.4. Recurrence and Repeat Resection of Colorectal Liver Metastases. Although liver resections are performed with curative intent, 60% will develop recurrent disease. Between 20–30% with recurrence after the first liver resection will have a disease which potentially allows a repeat liver resection. The criteria for surgery are relatively the same as for the initial resection. Survival rates and risk for complications and length of hospital stay are reported similar to that noted after the first resection [15, 25–29].

2. Patients and Methods

Medical data on consecutive patients that underwent liver resection due to colorectal adenocarcinoma metastases at the Department of Surgery, Skåne University Hospital, Sweden, during the period 1995–2009, was collected in a database. The information was taken from patients subjected to liver resection for CRLM. Follow-up data was also retrieved for patients who were referred from other hospitals. Four patients registered as having only local intervention actually had a formal liver resection performed and thus entered the database. The database includes a large variety of information, for example, age, intraoperative bleeding, operation time, and complications.

In total, 240 patients had liver resection due to CRLM during the period 1995–2009. Patients who were included in this study had CRLM and underwent a repeated liver resection during the period 1995–2009 at the Department of Surgery, Skåne University Hospital Lund, Sweden. We identified 32 patients, 30 out of which with one reresection of the liver and the other two had two reresections, thus making a total of 34 repeated liver resections. All patients were operated upon with curative intent. The 240 primary liver resections served as control group.

2.1. Statistical Methods. The tests used were Mann-Whitney U test, Fisher's exact test, Kruskal-Wallis test, and the Kaplan-Meier test.

3. Results

There was no 30-day operative mortality or postoperative hospital deaths following the 34 repeat resections. The median age at the primary resection was 66 years compared to 64 at the repeat resection (n.s.). Of the 240 primary resections, 91 were female compared to 14/34 at the repeat resection (38% versus 41%; $P = 0.710$). The primary resection was more often a large (hemihepatectomy or more than 3 segments) resection as compared to the repeat resections ($P < 0.0001$). The median intraoperative blood loss at repeat resection was 1850 mL, significantly higher as compared to the bleeding noted at primary liver resections (in median 1000 mL; $P = 0.014$). This was the fact even though smaller resections had significantly less bleeding compared to large resections ($P = 0.012$). The median hospital stay at a repeat resection was 8 days, that is, the same as at a primary resection ($P = 0.98$). There was no difference in the rate of complications ($P = 0.568$) between primary and repeat liver resections.

There was no difference in the number of patients that received neoadjuvant chemotherapy (within the month prior to the operation) or adjuvant chemotherapy ($P = 0.826$ and $P = 0.748$, resp.).

There was no difference in survival (Figure 1) between the two groups ($P = 0.556$), despite the fact that repeat

Table 1: Primary and repeated liver resection due to colorectal liver metastases—outcome data.

	Primary resection	Repeat resection	P value
Age	66 ± 10 years	64 ± 9 years	n.s.
Gender	62% males	59% males	n.s.
Bleeding	1000 ± 1786 mL	1850 ± 1833 mL	0.014
Length of stay	8 ± 8 days	8 ± 3 days	n.s.
Neoadjuvant chemo. administered	33%	31%	n.s.
Adjuvant chemo. administered	39%	36%	n.s.
Clavien grade II+	65%	71%	n.s.
2-year survival	76%	86%	n.s.

resections were less microscopically radical according to the PAD ($P = 0.046$). The tumours were slightly larger at the primary resection, though not significantly ($P = 0.108$).

Increased bleeding at the primary resection (>1000 mL) was associated with a higher risk for both complications ($P =< 0.0001$) and an increased length of stay ($P = 0.004$).

A higher ASA-class tended to increase the length of hospital stay ($P = 0.052$). When excluding ASA-class IV from the equation, the difference was not significant ($P = 0.12$). ASA-class did not significantly affect neither bleeding ($P = 0.092$) nor risk for complications ($P = 0.611$; Table 1).

4. Discussion

During the last decades, there has been great advancement in the field of management of colorectal liver cancer metastases, including recurrent disease. Repeated resections of the liver for colorectal liver metastases have in most studies proved to be beneficial, even rendering some improvement in cases with multiple metastases. Patients thus tolerated the surgical resection well despite a technically difficult operation on a liver potentially damaged by chemotherapy, and mortality and morbidity do not seem to be higher than that reported following a primary liver resection.

In our study, we noted that the intraoperative bleeding was significantly higher in repeated resections as compared to the primary resection. Previous studies [26] have shown similar results. A probable cause of the increased bleeding may be the altered anatomy, in and around the liver, with scar tissues and adhesions, which thus makes it more difficult to avoid an increase in the intraoperative bleeding. In addition, the repeat resections were more often atypical, suggesting less clear segmental limits, therefore making it an even more challenging operation.

The increased intraoperative bleeding in repeat liver resections did not have a negative effect on outcome considering the rate of complications and length of hospital stay. This was a little surprising, as a bleeding of more than 1000 mL in itself was a risk factor for both an increase in the rate of complications as well as length of stay. An explanation could be the fairly limited number of patients, thereby decreasing the possibilities to draw definite conclusions.

We also found that there were no differences between the two groups in terms of how many that received neoadjuvant or adjuvant chemotherapy. This can be interpreted that patients in both groups are treated equally from an oncological point of view and thereby has the same conditions when subjected to liver surgery. Overall, repeat liver resections are handled in a similar way as primary liver resections. By treating the two groups in the same way one can assume that experience accumulated from primary resections are also valid on repeat resections. This is important, as repeat resections are fairly limited in number, though the overall accumulating data support the safety and benefits of repeat liver resections [5, 30–32].

5. Conclusion

Repeated resections of the liver in patients with colorectal liver metastases are associated with an increase in intraoperative bleeding as compared to a primary liver resection. However, this had no effect on the outcome, on survival, rate of complications, or on length of hospital stay. Therefore, repeated liver resections for colorectal liver metastases represent a safe and worthwhile operation with results in similarity with those reported following primary liver resections.

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Clinical Study

Ion Chromatography Based Urine Amino Acid Profiling Applied for Diagnosis of Gastric Cancer

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Aim. Amino acid metabolism in cancer patients differs from that in healthy people. In the study, we performed urine-free amino acid profile of gastric cancer at different stages and health subjects to explore potential biomarkers for diagnosing or screening gastric cancer. *Methods*. Forty three urine samples were collected from inpatients and healthy adults who were divided into 4 groups. Healthy adults were in group A ($n = 15$), early gastric cancer inpatients in group B ($n = 7$), and advanced gastric cancer inpatients in group C ($n = 16$); in addition, two healthy adults and three advanced gastric cancer inpatients were in group D ($n = 5$) to test models. We performed urine amino acids profile of each group by applying ion chromatography (IC) technique and analyzed urine amino acids according to chromatogram of amino acids standard solution. The data we obtained were processed with statistical analysis. A diagnostic model was constructed to discriminate gastric cancer from healthy individuals and another diagnostic model for clinical staging by principal component analysis. Differentiation performance was validated by the area under the curve (AUC) of receiver-operating characteristic (ROC) curves. *Results*. The urine-free amino acid profile of gastric cancer patients changed to a certain degree compared with that of healthy adults. Compared with healthy adult group, the levels of valine, isoleucine, and leucine increased ($P < 0.05$), but the levels of histidine and methionine decreased ($P < 0.05$), and aspartate decreased significantly ($P < 0.01$). The urine amino acid profile was also different between early and advanced gastric cancer groups. Compared with early gastric cancer, the levels of isoleucine and valine decreased in advanced gastric cancer ($P < 0.05$). A diagnosis model constructed for gastric cancer with AUC value of 0.936 tested by group D showed that 4 samples could coincide with it. Another diagnosis model for clinical staging with an AUC value of 0.902 tested by 3 advanced gastric cancer inpatients of group D showed that all could coincide with the model. *Conclusions*. The noticeable differences of urine-free amino acid profiles between gastric cancer patients and healthy adults indicate that such amino acids as valine, isoleucine, leucine, methionine, histidine and aspartate are important metabolites in cell multiplication and gene expression during tumor growth and metastatic process. The study suggests that urine-free amino acid profiling is of potential value for screening or diagnosing gastric cancer.

1. Introduction

Gastric cancer is one of the most common malignancies and the second cause of cancer-associated death worldwide [1, 2]. The early diagnosis is very difficult because there are no specific symptoms at an early stage of gastric cancer, and early gastric cancer is typically small [3, 4]. Clinically, most gastric cancers were identified when they were at an advanced stage. Advanced gastric cancer which has a high mortality for its local and distant metastases does a great harm to human's health [4–8]. Up to now, we are not able to carry out any effective causal prophylaxis because the etiopathogenesis of gastric cancer is not defined [9, 10]; therefore, early diagnosis or screening is especially important to gastric cancer. Although endoscopy combining biopsy is a fairly mature method now, the rate of diagnosis is still relying on the experience of endoscopists and gastrointestinal pathologist [11, 12]. The serologic tests for gastric cancer such as CEA have little diagnosis value for their lower specificity and sensibility [13–15].

Amino acids in human body include exogenous and endogenous amino acids. They are distributed allover the body to participate in metabolism, called amino acid metabolic pool. Endogenous amino acids which are produced from protein degradation in tissue can participate in varied physiological adjustments, such as gene expression, cell multiplication, and inflammatory reaction. The fast speed of cell multiplication and prosperity metabolism is characteristic of malignancy [16]. So malignant cells need a large number of amino acids from amino acid metabolic pool to synthesize protein and nucleic acids. An abnormal plasma-free amino acid (PFAA) profile might be presented for the total reflection of cancer-induced protein metabolism in tumors, skeletal muscle, and liver in cancer patients. Some studies indicated that amino acid metabolism is not the same in different types of malignant tumors. Kubota et al. [17] studied PFAA concentrations in 58 cancer patients, including 22 breast cancer, 24 gastrointestinal cancer, and 12 head and neck cancer. The results showed that the seven amino acids (glutamine, threonine, histidine, cysteine, alanine, arginine, and ornithine) had a close link with specific cancers, indicating that PFAA profiles correlate with the organ-site origin among the three different malignant tumors. Reduction of gluconeogenic amino acids has been observed in early tumor growth in an animal study [18]. This reduction occurred as early as 6 days after tumor cell inoculation, when the tumor was not detectable. The staging of cancer characterized by tumor size, depth of invasion, and metastasis is considered to be related with the PFAA profile [19].

In recent years, metabonomics as a branch of systems biology has developed rapidly. Now, it has been established as an extremely powerful analytical tool and hence found successful applications in many research areas including molecular pathology and physiology, drug efficacy and toxicity, gene modifications and functional genomics, environmental sciences, and disease diagnoses [20–26]. In oncology, metabonomics can apply various advanced techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography/mass spectrometry (HPLC/MS, and LC/MS/MS), Fourier-transform infrared (FT/IR) spectroscopy, and gas chromatography/mass spectrometry (GC/MS) to detect and measure low-molecular-weight metabolites in animal and human body fluid (blood, urine, etc.) [27–32]. Metabonomics combining chemometrics can reveal metabolic changes in malignant tumors and show powerful values in clinical study.

Ion chromatography (IC) has been proven to be an excellent metabonomic tool and applied in metabolites identification and quantification based on its convenience, high sensitivity, peak resolution, and reproducibility. In this study, we used IC to detect urine-free amino acids profiles of early gastric cancer, advanced gastric cancer, and health people. Amino acids in the human body undergo interdependent regulation; comparing single amino acid concentration between patients and controls might be insufficient to elucidate amino acid changes associated with cancer development. Therefore, differences in amino acid profiles from the three groups were characterized by principal components analysis (PCA) in the present study. Based on pattern results, we tried to construct a diagnostic model to discriminate gastric cancer from healthy individuals and another diagnosis model for clinical staging.

2. Materials and Methods

2.1. Materials. All standards and samples were prepared with deionized water (Labconco, Kansas City, MO, USA). Sodium hydroxide (NaOH) (50%, w/w) was purchased from Fisher Scientific (Hampton, NH, USA). Omithine (≥99.5%), cystine (≥99%), sodium acetate (≥98%), and amino acid standard solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA). Spermidine trihydrochloride (>98%) and spermine tetrahydrochloride (≥99%) were purchased from Calbiochem (San Diego, CA, USA).

2.2. Sample Collection and Preservation. Twenty six inpatients, aged 53 to 86 years and diagnosed with gastric cancer, were categorized according to endoscopic examination coupled with histopathological features and stages according to the seventh edition of the International Union Against Cancer (UICC) TNM: stages I and II (early-stage cancer), 7 patients (female/male, 3/4), aged 53 to 86 years (the median age was 72 years old); stages III and IV (advanced-stage cancer), 19 patients (female/male, 9/10), aged 54 to 84 years (the median age was 76 years old). Patients enrolled in this research were not on any medication before sample collection. The clinical diagnosis and pathological reports of all the patients were obtained from the hospital. Seventeen healthy subjects (female/male, 8/9), aged 50 to 86 years (the median age was 68 years old), were selected by a routine physical examination including endoscopy, and any subjects with chemotherapy, kidney disease, and endocrine disorders were excluded. Urine samples were collected in the morning before breakfast from a total of 26 gastric cancer patients and 17 healthy volunteers at Shanghai Sixth Hospital, Medical College of Shanghai Jiao Tong University (Shanghai, China). All the patients and subjects were Han Chinese living in China and had normal nutritional status. The protocol was approved by the Shanghai Sixth Hospital Institutional Review Board, and all participants gave informed consent before they were involved in the study. In this study, we used IC to detect urine free amino acids profiles of gastric cancer and health of people. To study urine-free amino acids profiles for screening or diagnosing gastric cancer especially for early gastric cancer, urine samples were divided into 3 groups.

2.3. Ion Chromatography. The chromatography system consisted of a Dionex ICS-3000 Reagent-Free TM Ion Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) with a DP-3000 dual gradient pump, a DC-3000 detector compartment with a conductivity cell and an electrochemical cell, an EG-3000 eluent generator with an EluGen EGC II MSA cartridge, and an AS autosampler.

Amino acids were separated with an AminoPac PA10Pac CS18 (250 mm × 2 mm I.D., Dionex Corporation) analytical column and its respective guard column, CG18 (50 mm × 2 mm I.D.) with a flow rate of 0.25 mL/min and

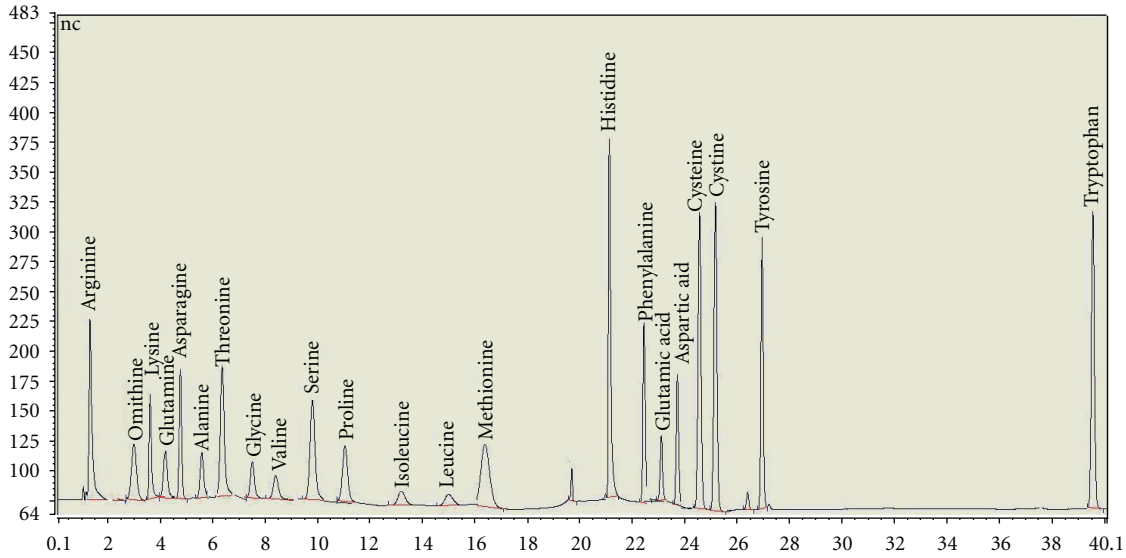

Figure 1: IC chromatogram of 22 amino acids standard solution: (1) arginine; (2) omithine; (3) Lysine; (4) glutamine; (5) asparagine; (6) alanine; (7) threonine; (8) glycine; (9) valine; (10) serine; (11) proline; (12) isoleucine; (13) leucine; (14) methionine; (15) histidine; (16) phenylalanine; (17) glutamic acid; (18) aspartic aid; (19) cysteine; (20) cystine; (21) tyrosine; (22) tryptophan.

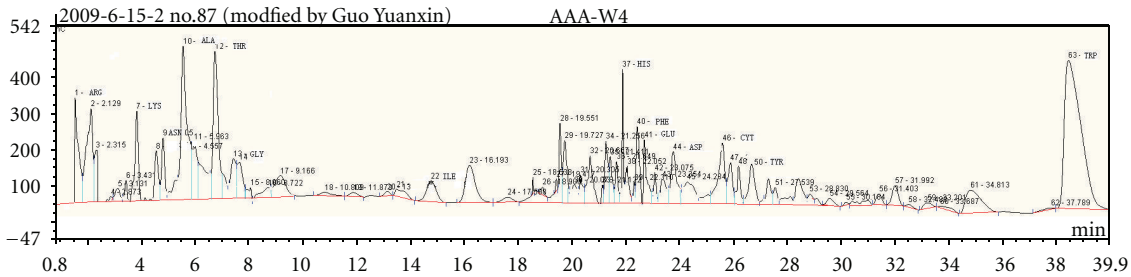

Figure 2: Total ion current of a normal subject urine.

a thermostated temperature of 30°C. A CSRS ULTRA II (2 mm) self-regenerating suppressor operating at 40 mA in the external water mode was used for suppressed conductivity detection. A 25 μL sample injection volume was used throughout the experiment. Operating backpressure was less than 3,000 psi. The gradient elution conditions consisted of deionized water from 0 to 42 min, 40 mM to 200 mM NaOH from 0 to 42 min, and 400 mM to 700 mM sodium acetate from 18 to 42 min.

2.4. Statistical Analysis. After the chromatographic peak area was normalized, the PCA analysis was done to construct urine amino acid metabolic profile of different stages of gastric cancer patients and control subjects. All data were expressed as mean ± SD. Statistical analysis was performed using Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chromatogram of 22 Amino Acid Standard Solution. From Figure 1, it was showed that 22 amino acids were all separated effectively in 40 minutes. The concentration of standard solution was 8 μM.

3.2. Total Ion Current Chromatogram for Health Adult Group and Gastric Cancer Group. As can be seen from Figures 2 and 3, the urine chromatograms of health adult group and gastric cancer group detected by IC showed that the total ion current (TIC) peaks of two groups were different. At the same retention time, peak size and peak height were different between groups.

After IC analysis, each sample was represented by a TIC, and the peak areas of amino acids were integrated. We qualitatively analyzed 22 amino acid chromatogram of each urine sample according to the chromatogram of 22 amino acid standard solution, and the peak-area ratio of each compound to a corresponding internal standard was calculated as the response by using peaknet6 software. Statistical analysis was performed using Wilcoxon rank sum test. Table 1 showed that the urine-free amino acid profiles of gastric cancer changed to a certain degree compared with healthy adult subjects. Compared with healthy adult group, the levels of valine, isoleucine, and leucine increased

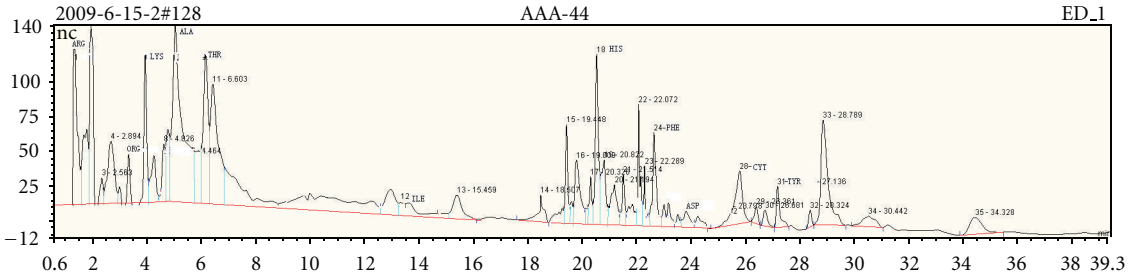

Figure 3: Total ion current of a gastric cancer inpatient urine.

Table 1: Urine amino acid profiles of healthy adult group and gastric cancer group [R = (healthy adult group − gastric cancer group)/gastric cancer group].

Amino acids	Healthy group (group A)	Gastric cancer (group B + C)	R	P
Isoleucine	1.640 ± 0.771	2.539 ± 1.661	−0.354	0.024
Leucine	2.289 ± 1.162	3.426 ± 2.376	−0.332	0.047
Valine	0.789 ± 0.863	1.535 ± 1.128	−0.486	0.033
Methionine	3.551 ± 1.931	1.997 ± 1.249	0.778	0.011
Histidine	3.292 ± 1.885	1.882 ± 0.837	0.749	0.014
Aspartic aid	3.312 ± 1.594	1.620 ± 1.468	1.044	0.001

($P < 0.05$), but histidine and methionine decreased ($P < 0.05$), and aspartate decreased significantly ($P < 0.01$) in gastric cancer patients.

When we compared urine amino acid profiles of early gastric cancer to advanced gastric cancer, valine level decreased ($P < 0.05$) and isoleucine level remarkably decreased in advanced gastric cancer ($P < 0.01$) as shown in Table 2.

3.3. Pattern Recognition. SPSS16.0 software is used for PCA analysis of the data; PCA scores plot showed that different urine samples (healthy control and gastric cancer groups) were scattered into two different regions (Figure 4). ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. The sensitivity and specificity trade-offs were summarized for each variable with the area under the curve (AUC). The AUC value of this PCA model was 0.936 (Figure 5), which demonstrated a good differential value for gastric cancer.

We make PCA according to PC1 and PC2 of five tested samples to test the diagnosis model for gastric cancer (Figure 6), and we can see that two cases of normal samples are all in the normal region, and that two cases of gastric cancer samples are in the cancer region except one case of gastric cancer is in the noncancer region.

PCA was also performed to differentiate between early and advanced gastric cancer groups. Figure 7 showed that most urine samples from early gastric cancer were separated from advanced cancer samples. This PCA model was also validated by ROC analysis (AUC = 0.902, Figure 8).

There were no early gastric cancer samples in tested samples, so we just take three cases of advanced gastric cancer samples to PCA (Figure 9), and we can see that 3 cases of advanced gastric cancer samples are all in the advanced cancer region.

4. Discussion

In the current study, we performed urine amino acid profile to identify marker metabolites. Some amino acids were differentially expressed in patients with gastric cancer and control subjects. Diagnosis model for gastric cancer which was tested by a small-scale sample showed its potential value in clinical diagnosis. The high AUC value indicated that the PCA model was robust in the discrimination. Another diagnosis model for gastric cancer staging which was also tested by a small-scale sample was of potential value in clinical diagnosis. Figure 7 showed that some of samples from early gastric cancer were located at advanced gastric cancer samples. Cancer can progress quantitatively or qualitatively, and these patients may be in the intermediate stage from early gastric cancer to advanced gastric cancer.

It has been reported that amino metabolism is remarkably perturbed in cancer cells [4, 33], and urine amino acid profiles are also altered [12, 34–36]. Changes in amino acid metabolism and an increase in gluconeogenesis have been well documented in cancer patients [35, 36]. In the present study, the model identified patients at early stage of gastric cancer and advanced gastric cancer, suggesting that the urine amino acid profiling is useful for diagnosis of gastric cancer.

The results showed that the isoleucine, leucine, and valine levels in urine of patients with gastric cancer were significantly higher than those in normal controls. As malignant tumors grow rapidly, they need a large number of amino acids from the metabolism pool as a substrate

Table 2: Urine amino acid profiles of early and advanced gastric cancer groups [R = (early gastric cancer group − advanced gastric cancer group)/advanced gastric cancer group].

Amino acids	Early gastric cancer group	Advanced gastric cancer group	R	P
Isoleucine	3.946 ± 1.982	2.023 ± 1.214	0.951	0.006
Valine	2.568 ± 1.506	1.155 ± 0.665	1.223	0.048

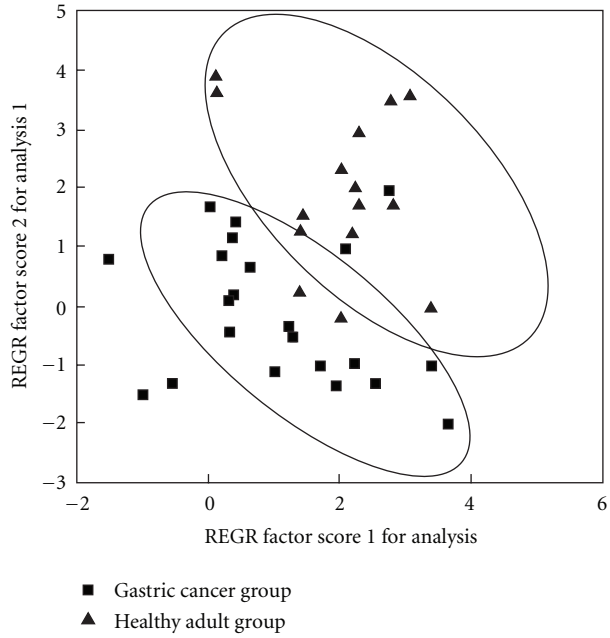

Figure 4: PCA scores plot of urine-free amino acids in healthy adult group and gastric cancer group (diagnosis model for gastric cancer).

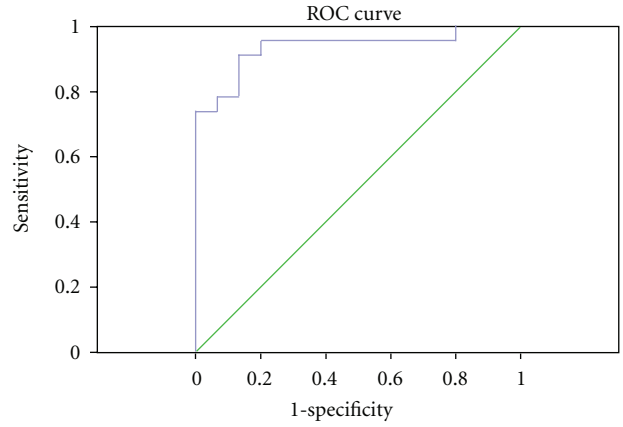

Figure 5: ROC of diagnosis model for gastric cancer (AUC = 0.936).

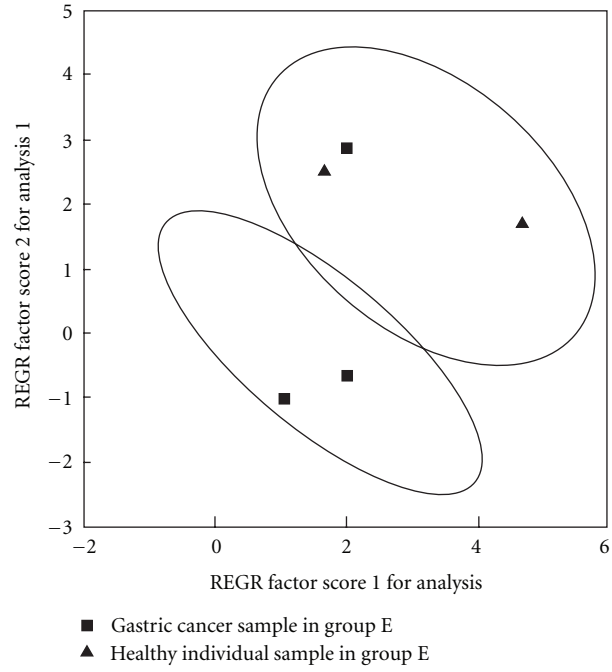

Figure 6: PCA of urine-free amino acids in group E (test result of diagnosis model for gastric cancer).

for synthesis of proteins and nucleic acids and other substances. The metabolism of amino acids in body's muscle tissue is above 50% of the total metabolism, and the catabolism of branched-chain amino acids (BCAA), such as valine, leucine, and isoleucine, is mainly involved in skeletal muscle. So tumor tissues have high demand on the BCAA. Many experiments showed that patients with malignant tumors, including gastric cancer, tended to be high metabolic. Isoleucine is a glucogenic and ketogenic amino acid which decomposed into acetyl-coenzyme A and succinate-coenzyme A, the important materials in the citric acid cycle, which were greatly required during the gluconeogenesis [19], so the level of isoleucine in the gastric cancer patients is higher than the normal group. Most cancer cells predominantly used amino acids to produce more energy by glycolysis but not oxidative phosphorylation via the tricarboxylic acid (TCA) cycle [33, 37]. Valine is a glucogenic amino acid, and leucine is a ketogenic amino acid. So, the levels of isoleucine, leucine, and valine which are important to the process of gluconeogenesis in the cancer patients are higher. As tumor cells have an active nucleic acid metabolism, histidine was substantially absorbted into tumor tissue, and consumption significantly increased, leading to its decline in body. Moreover, the metabolism of specific amino acids is known to be related to specific organs, such as muscle, or liver, and changes in the levels of amino acids are affected by their metabolism in organs of the body. Some amino acids were reported to correlate with specific cancers [17, 38]. Therefore, profiling plasma or urine amino

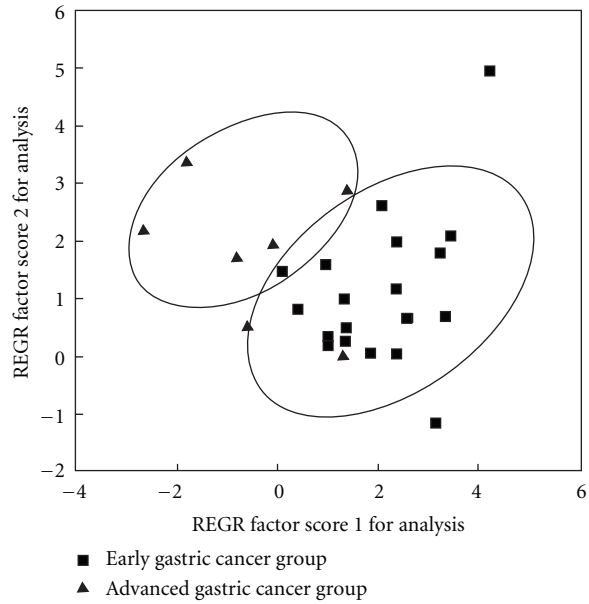

Figure 7: PCA of urine free amino acids in early and advanced gastric cancer group (diagnosis model for clinical staging).

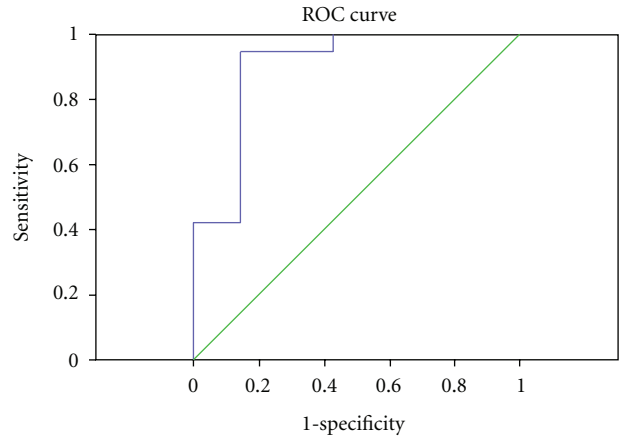

Figure 8: ROC of diagnosis model for clinical staging (AUC = 0.902).

acids can detect the metabolic alterations in specific organs, which may be applied in early cancer diagnosis.

From the present study, the isoleucine and valine levels in urine of patients with early gastric cancer were slightly higher than those of advanced gastric cancer. It may be because protein was excessively consumed in advanced cancer, which leads to a lower level of valine than in early cancer group. Yamanaka et al. made a hypothesis: the increased obstacles in muscle tissue protein improved the glucose gluconeogenesis in hepatic and increased the oxidation of BCAA in muscle, which was the main mechanism for the rise of BCAA levels in the patients of early gastric cancer; tumor can stop the further development of this increased muscle protein barrier, and then intravenous BCAA and EAA levels may decline.

In conclusion, MS-based techniques, such as liquid chromatography/mass spectrometry (LC/MS) including ion

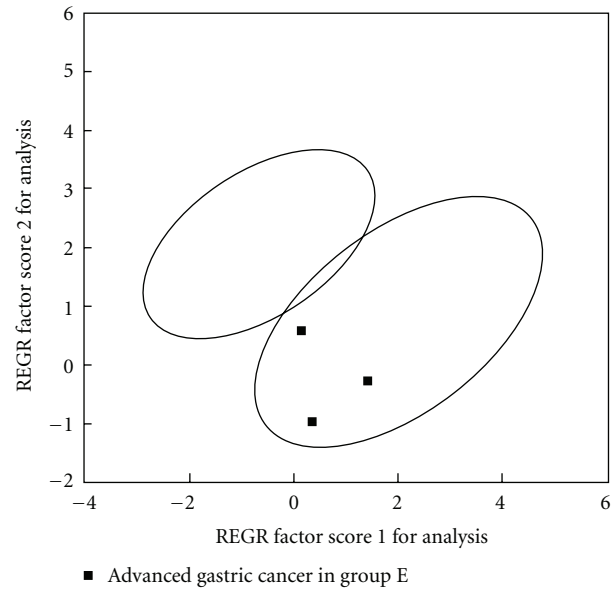

Figure 9: PCA of urine free amino acids of 3 advanced gastric cancer in group E (test result of diagnosis model for clinical staging).

chromatography and gas chromatography/mass spectrometry (GC/MS), are very important tools in the diagnosis of many diseases. In this study, we found that amino acid balance in stomach cancer patients was significantly different from the healthy individuals, also there were differences between early gastric cancer and advanced gastric cancer. We believe that ion chromatography technique has great potential in the early diagnosis or screening of diseases especially gastric cancer and is worthy of further evaluation and research.

Conflict of Interests

The authors state that there is no conflict of interests.

Acknowledgments

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Research Article

Effect of Non-Anticoagulant N-Desulfated Heparin on Basic Fibroblast Growth Factor Expression, Angiogenesis, and Metastasis of Gastric Carcinoma In Vitro and In Vivo

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Objective. The present study was performed to investigate the effect of N-desulfated heparin on basic fibroblast growth factor (bFGF) expression, tumor angiogenesis and metastasis of gastric carcinoma. *Methods*. Human gastric cancer SGC-7901 tissues were orthotopically implanted into the stomach of NOD SCID mice. Twenty mice were randomly divided into two groups which received either intravenous injection of 0.9% NaCl solution (normal saline group) or 10 mg/kg N-desulfated heparin (N-desulfated heparin group) twice weekly for three weeks. In vitro, human gastric carcinoma SGC-7901 cells were treated with N-desulfated heparin in different concentration (0.1 mg/mL, 1 mg/mL, N-desulfated heparin group), and treated with medium (control group). *Results*. In vivo, the tumor metastasis rates were 9/10 in normal saline group and 2/10 in N-desulfated heparin group ($P < 0.05$). The intratumoral microvessel density was higher in normal saline group than in N-desulfated heparin group ($P < 0.05$). bFGF expression in gastric tissue was inhibited by N-desulfated heparin ($P < 0.05$). There was no bleeding in N-desulfated heparin group. In vitro, N-desulfated heparin inhibited significantly bFGF protein and mRNA expression of gastric carcinoma cells ($P < 0.05$). *Conclusions*. N-desulfated heparin can inhibit the metastasis of gastric cancer through inhibiting tumor bFGF expression and tumor angiogenesis with no obvious anticoagulant activity.

1. Introduction

Gastric cancer is the common alimentary tract cancer in China in terms of incidence. It is one of the malignancies that do serious harm to people's health with a high mortality and are short of effective therapeutic methods. Recent studies have showed that angiogenesis plays a crucial role in tumor growth and metastasis. Angiogenesis, which is the process by which new blood vessels develop from preexisting vessels, is governed by a very complex network of opposing signals that, under normal physiological conditions, are elicited by various highly regulated angiogenesis stimulators and inhibitors [1]. Angiogenesis is essential for tumor growth beyond a few millimeters in diameter because of the tumor's requirement for a network of blood vessels to deliver oxygen and nutrients and to remove waste products of metabolism. During tumor-associated angiogenesis, the balance of angiogenesis stimulators and inhibitors is tipped in favor of angiogenesis by hypoxia-inducible factor-1 gene expression [2]. Inhibition of angiogenesis can control tumor metastasis and improve the prognosis [3–6]. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are the main factors promoting angiogenesis [7, 8]. Even though VEGF is a primary mediator of angiogenic responses, bFGF is more potent than VEGF for stimulating the vascular endothelial mitogenesis. Anti-VEGF therapy is effective in inhibiting angiogenesis and metastasis of tumor [9, 10]. Heparin, a highly sulfated proteoglycan, has been extensively used as an anticoagulant drug for a long time. Aside from its anticoagulant action, heparin binds to various growth factors, cytokines, and extracellular proteins and consequently is able to affect migration of cancer cells and angiogenesis in tumors. The potential anticancer activity of

heparins is supported by data from in vitro and experimental studies [11]. Stevenson et al. [12] has reported that heparin primarily reduces metastatic disease by inhibiting P- and L-selectin interactions. However, clinical use of heparin in treatment of tumor is limited by its strong anticoagulant activity, which may cause severe bleeding complications. Chemically modified heparin shows a significantly reduced anticoagulant activity and enhanced ability to interact with FGF, VEGF, and hepatocyte growth factor, which are known to stimulate angiogenesis [13]. In this study, we investigated the effect of N-desulfated heparin on bFGF expression, angiogenesis, and tumor metastasis in vitro and in vivo.

2. Materials and Methods

2.1. Materials. Goat anti-human CD34 antibody and goat anti-human bFGF antibody were obtained from Santa Cruz Biotechnical Company. bFGF probe for real-time PCR was provided by Daan Gene Company of Zhongshan University. Human gastric adenocarcinoma SGC-7901 cell line was obtained from the Cell Biology Institute of Chinese Academy of Sciences, Shanghai.

2.2. Gastric Cancer Cell Cultivation. Human gastric cancer SGC-7901 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 37°C in a humidified atmosphere containing 5% CO_2. Human gastric cancer SGC-7901 cells were suspended at a concentration of 3×10^6/10 mL. The different concentrations (0.1 mg/mL, 1 mg/mL) of N-desulfated heparin and medium were added to the cells. Cells were harvested after 12 h and 24 h. Cells were washed once with PBS and scraped into a wash buffer. The cells were washed in the buffer, homogenized in 150 μL cell lysis buffer, and incubated on ice for 30 min. The supernatants were recovered and snap-frozen in liquid nitrogen and stored at −80°C.

2.3. Enzyme-Linked Immunosorbent Assay. A 96-well microwell plate (Nunc, Kamstrup, Denmark) was coated with 100 μL of a 1 : 4000 dilution of purified IgY-anti bFGF in sodium bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plate was washed twice with PBS containing 0.05% Tween 20 (PBST), and 100 μL of recombinant bFGF-p24 was added, diluted 1 : 100 in 5 g/L pluripeptone, 3 g/L meat extract containing 0.05% Tween 20 (PMET). Incubation was performed at 37°C for 120 min. The microwell plate was washed four times with PBST and incubated at 37°C for 60 min with 100 μL monoclonal antibody against p24 (VMRD), diluted 1 : 2000 in PMET. The plate was washed four times with PBST and incubated 60 min at 37°C with 100 μL of anti-mouse-peroxidase diluted 1 : 4000 in PBST. The plate was washed again with PBS and detected with 90 μL of tetramethylbenzidine (TMB) for 5~10 min at 37°C. The reaction was stopped by addition of 30 μL of 2 mol/L H_2SO4. Optical density value (OD) was measured at a wavelength of 492 nm. Each assay was performed three times, and the average results were calculated, using Ascent software for Multiskan reader.

2.4. Animal Model. Male NOD severe combined immune deficiency (SCID) mice were obtained from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animal experimental procedures were performed according to the relative ethical regulations for the care and use of laboratory animals of our university. Animals used were 6 weeks old and weighed 20–25 g. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Animal models were made using orthotopic implantation of histologically intact tissue of human gastric carcinoma [14]. Tumors were resected aseptically. Necrotic tissues were cut, and the remaining healthy tumor tissues were scissor minced into pieces (about 5 mm × 7 mm in diameter) in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to be 150 mg. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision was made through the left upper abdominal pararectal line. Then, peritoneal cavity was carefully exposed, and a part of serosal membrane in the middle of the greater curvature of stomach was mechanically injured using scissors. A tumor piece of 150 mg was fixed on each injured site of the serosal surface. The stomach was returned to the peritoneal cavity, and the abdominal wall and skin were closed. After metastatic models were made, the mice were randomly divided into N-desulfated heparin group ($n = 10$) and normal saline group ($n = 10$). One week after operation, the mice in N-desulfated heparin group received i.v. injections of N-desulfated heparin (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, 10 mg/kg·d) twice weekly for 3 weeks. The mice in normal saline group received i.v. injections of normal saline (100 μL) twice weekly for 3 weeks. The mice were weighed twice weekly.

2.5. Sample Collection and Pathological Examination. All animals were sacrificed at week 6 after implantation. An incision was made through the abdominal wall, and then peritoneal cavity was carefully exposed. Tumors growing on the stomach wall were removed and fixed in 10% formalin and processed for routine paraffin embedding. Tissues from all organs and lymph nodes were collected and fixed in 10% formalin and processed for routine paraffin embedding after careful macroscopic examination. Four-micron-thick sections were stained with hematoxylin and eosin and evaluated histologically for liver metastasis or lymph node metastasis or other organ metastasis under microscope.

2.6. Mean Microvascular Density of Tumor (MVD). Immunostaining was performed using a labeled streptavidin biotin method. Four-micron-thick sections were deparaffinized in xylene and rehydrated with graded alcohol. Immunohistochemical staining was carried out to detect CD34 expression following the manufacturer's protocol. The concentration of anti-CD34 antibody was 1 : 300. MVD (CD34-positive microvessels) was calculated under 200-fold microscope. The modified Weidner's method was used for the evaluation of MVD according to CD34 endothelial cell immunostaining.

For the microvessel counting, positive stainings for MVD in 5 most highly vascularized areas in each section were counted in 200 × fields. MVD was expressed as average of the microvessel count in the areas.

2.7. Detection of bFGF Expression. Immunostaining was performed using a labeled streptavidin biotin method. Four-micron-thick sections were deparaffinized in xylene and rehydrated with graded alcohol. Immunohistochemical staining was carried out to detect bFGF expression following the manufacturer's protocol. The concentration of anti-bFGF antibody was 1 : 60. Positive cells under 10% were defined as positive +, over 10% as positive ++. Positive expression was defined as positive + or positive ++.

2.8. Detection of bFGF mRNA Expression. bFGF primers and probe used are bFGF f: 5′-GTCACGGAAATACTCCAGTTG, bFGF r: 5′-CCGTTTTGGATCCGAGTTTATACT-3, bFGF probe: 5′-TGTGGCACTGAAACGAACTGGG-3. bFGF mRNA was isolated by method of Trizol. Synthesis of the first strand cDNA was performed according to the instructions delivered with reverse transcription kit, using human bFGF antisense strand primers and reverse transcriptase. After 1 h incubation at 37°C, samples were heat inactivated for 3 min at 95°C and kept at −80°C until use. Aliquots of 5 μL of cDNA were amplified in a final volume of 50 μL using PCR buffer at the presence of 1 μL of Taq DNA polymerase and 0.5 μL of bFGF probe. Samples were amplified at 93°C for 2 min, at 93°C for 0.5 min, and at 55°C for 1 min followed by 40 cycles. Real-time PCR was carried out in an automated real-time PCR cycler (American ABI 7000).

2.9. Statistical Analysis. All data were expressed as mean ± SD. Student's t-test and χ^2 precise method were used to determine changes in different groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of N-Desulfated Heparin on Metastasis of Human Gastric Cancer. All mice developed localized tumors at the implanted site, which were poorly differentiated adenocarcinomas under microscope. Tumor growth did not differ significantly between the animals treated with normal saline or with N-desulfated heparin. Of the 10 animals treated with normal saline, 9 developed metastatic tumors in regional lymph nodes, 8 in liver, and 6 in other organs. However, after the mice were treated with N-desulfated heparin for 3 week, metastasis of tumor was inhibited significantly. Of the 10 animals treated with N-desulfated heparin, 2 developed metastatic tumors in liver. The metastatic rate was higher in mice treated with normal saline than in those treated with N-desulfated heparin (90% versus 20%, $P < 0.05$). N-desulfated heparin had no significant effect on body changes in NOD SCID mice. No bleeding complications were found in N-desulfated heparin group.

Table 1: Effect of N-desulfated heparin on bFGF expression in gastric cancer in vivo (mean ± SD).

Groups	n	−	+	++	Positive rate (%)
Normal saline group	10	1	2	7	90
N-desulfated heparin group	10	8	1	1	20

N-desulfated heparin group versus normal saline group, $P < 0.05$.

Table 2: bFGF expression of gastric cancer SGC-7901 cells in 0.1 mg/mL, 1 mg/mL N-desulfated heparin groups, and control group (pg/mL, $\overline{x} \pm s$, $n = 3$).

Groups	12 hr	24 hr
Control group	17.724 ± 0.173	19.690 ± 0.111*
0.1 mg/mL N-desulfated heparin group	15.313 ± 0.394$^{\triangle}$	13.301 ± 0.358$^{\triangle *}$
1 mg/mL N-desulfated heparin group	12.173 ± 0.063$^{\triangle \#}$	11.174 ± 0.286$^{\triangle *\#}$

*24 hr versus 12 hr, $P < 0.05$; $^{\triangle}$different concentrations (0.1 mg/mL, 1 mg/mL) of N-desulfated heparin versus control group, $P < 0.05$; $^{\#}$1 mg/mL N-desulfated heparin group versus 0.1 mg/mL N-desulfated heparin group, $P < 0.05$.

3.2. Effect of N-Desulfated Heparin on MVD. In normal saline-treated mice, many CD34 positively stained vessels were diffusely located and formed tube-like structures in tumor. However, they were almost absent in N-desulfated heparin-treated mice. The MVD was significantly lower in N-desulfated heparin-treated mice than in normal saline-treated mice (4.7 ± 1.8 versus 9.1 ± 3.4, $t = 3.617$, $P < 0.05$).

3.3. Effect of N-Desulfated Heparin on bFGF Protein Expression. Under microscope, bFGF positive immunostaining was found in cytoplasm of cancer cells. The rate of bFGF positive expression was higher in normal saline group than in N-desulfated heparin group ($P < 0.05$, Table 1).

3.4. Effect of N-Desulfated Heparin on bFGF mRNA Expression in Gastric Tissue of NOD SCID Mice. bFGF mRNA expression in gastric tissue of NOD-SCID mice detected by real-time PCR was higher in normal saline group than in N-desulfated heparin group (ct value 19.51 ± 1.01 versus 22.55 ± 1.36, $P < 0.05$).

3.5. Effects of N-Desulfated Heparin on bFGF Protein Expression In Vitro. The bFGF expression of human gastric cells was significantly increased with the extension of time in vitro (12 h versus 24 h, $P < 0.05$). With 0.1 mg/mL or 1 mg/mL N-desulfated heparin for 12 h and 24 h, bFGF expression was decreased significantly compared with control group (Table 2, $P < 0.05$). In 0.1 mg/mL and 1 mg/mL N-desulfated heparin groups, bFGF expression was decreased significantly at 24 h than at 12 h ($P < 0.05$). The results indicated that the inhibitory effect of N-desulfated heparin on bFGF expression of human gastric carcinoma cells was dose and time dependent.

Table 3: bFGF mRNA expression of gastric cancer SGC-7901 cell in different concentrations (0.1 mg/mL, 1 mg/mL) of N-desulfated heparin groups and control group (ct value, $\bar{x} \pm s$, $n = 3$).

	12 hr	24 hr
Control group	25.956 ± 0.505	$24.490 \pm 0.145^{*}$
0.1 mg/mL N-desulfated heparin group	$30.923 \pm 0.612^{\Delta}$	$32.493 \pm 0.358^{\Delta}$
1 mg/mL N-desulfated heparin group	$33.826 \pm 0.349^{\Delta\#}$	$35.446 \pm 0.299^{\Delta *\#*}$

*24 hr versus 12 hr, $P < 0.05$; $^{\Delta}$different concentrations (0.1 mg/mL, 1 mg/mL) of N-desulfated heparin versus control group, $P < 0.05$; $^{\#}$1 mg/mL N-desulfated heparin group versus 0.1 mg/mL N-desulfated heparin group, $P < 0.05$.

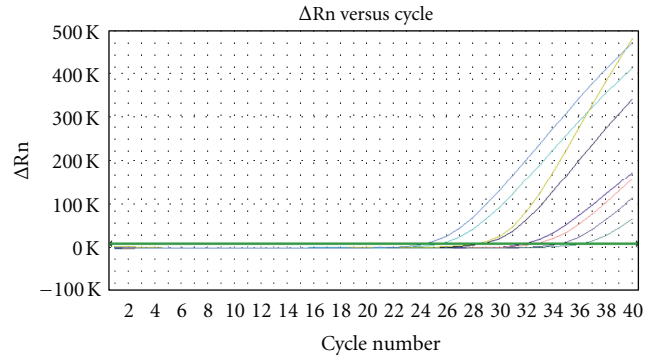

Figure 1: bFGF mRNA expression of human gastric cancer SGC-7901 cell in 0.1 mg/mL, 1 mg/mL N-desulfated heparin groups, and control group.

3.6. Effect of N-Desulfated Heparin on bFGF mRNA Expression of Gastric Cancer SGC-7901 Cells. In each of N-desulfated heparin groups, bFGF mRNA expression was decreased compared with control group ($P < 0.05$). The higher ct values mean the lower concentration of bFGF mRNA. The inhibitory effect of N-desulfated heparin on bFGF mRNA expression of human gastric carcinoma cells in vitro was associated with doses. Table 3 showed that the ct values in 0.1 mg/mL N-desulfated heparin groups were higher than in control group ($P < 0.05$) and that the ct values were higher in 1 mg/mL N-desulfated heparin group compared with 0.1 mg/mL N-desulfated heparin group ($P < 0.05$), suggesting that it had dose-dependent effects. In the same N-desulfated heparin concentration, the expression of bFGF mRNA at 24 h was lower than at 12 h ($P < 0.05$, 1 mg/mL N-desulfated heparin group), suggesting that the inhibition of N-desulfated heparin on bFGF mRNA expression of human gastric carcinoma cells had time-dependent effects (Figure 1, Table 3, $P < 0.05$).

4. Discussion

Tumor invasion and metastasis is a multistep process that promotes the spread of the cancer from primary sites to distant locations. Recent studies have showed that angiogenesis is a critical determinant of solid tumor metastasis, and antiangiogenic therapy plays an important role in improving prognosis of patients with gastric carcinoma [15–17]. VEGF represents a target for antiangiogenic therapies in a wide spectrum of diseases, including cancer. As a novel strategy to generate nonanticoagulant antiangiogenic substances exploiting binding to VEGF while preventing receptor engagement, Pisano et al. [18] assessed the VEGF-antagonist activity of a low-molecular-weight (LMW) compound generated by depolymerization of an undersulfated glycol-split heparin derivative. Unlike heparin, it was unable to present 125I-VEGF165 to its high-affinity receptors in endothelial cells and inhibited VEGF165-induced neovascularization in the chick embryo chorioallantoic membrane. Therefore, undersulfated, LMW glycol-split heparins may provide the basis for the design of novel nonanticoagulant angiostatic compounds. In addition, it has been reported that undersulfated and glycol-split heparins have endowed with antiangiogenic activity. Heparin binds to bFGF and promotes the formation of ternary complexes with endothelial cell surface receptors, inducing an angiogenic response [19]. N-desulfated heparin, a modified heparin, is known to have more significantly reduced anticoagulant activity (1/76 of heparin) than O-desulfated heparin (5%–30% of heparin) or N-acetylated heparin (10% of heparin) [20]. However, there is no report so far on the effect of N-desulfated heparin on tumor metastasis. Therefore, the effect of N-desulfated heparin on tumor metastasis, angiogenesis, and bFGF expression was observed in mouse model of orthotopic implantation of human gastric carcinoma tissue.

In the present study, tumor metastasis was inhibited significantly by N-desulfated heparin. To evaluate the effect of N-desulfated heparin on angiogenesis, immunohistochemical staining of CD34 in tumors was carried out. The results showed that N-desulfated heparin significantly inhibited angiogenesis in these tumors. Lee et al. [21] generated LHD or orally active heparin using low-molecular-weight heparin (LMWH) and deoxycholic acid that could be effectively absorbed in the gastrointestinal tract, making it an attractive candidate as an oral drug for antiangiogenic cancer therapy. Heparin oligosaccharides may be an inhibitor of the biological activity of bFGF on Caco-2 cells [22]. Norrby [23] found that 2.5 kDa and 5.0 kDa heparin fragments could specifically inhibit microvessel sprouting and network formation in VEGF165-mediated mammalian angiogenesis. Ono et al. [24] demonstrated that periodate-treated, nonanticoagulant heparin-carrying polystyrene (NAC-HCPS) affected angiogenesis and inhibited subcutaneous-induced tumour growth and metastasis to the lung. Mousa and Mohamed [25] have demonstrated antiangiogenic activity of the low-molecular-weight heparin, tinzaparin. Naggi et al. [13] has reported that N-acetylated and glycol-split heparins are potential antiangiogenic and antimetastatic agents which are more effective than unmodified heparin, suggesting that N-desulfated heparin can inhibit tumor metastasis by inhibiting angiogenesis.

bFGF is a ubiquitously expressed polypeptide growth factor that is normally sequestered in the extracellular matrix of healthy tissues [26]. It is also expressed by many human

cancer cells, including prostate carcinoma and melanoma cells and is believed to be important for the formation of tumor vasculature [27, 28].

In this study, the rate of bFGF positive expression was higher in normal saline group than in N-desulfated heparin group and bFGF mRNA expression was higher in normal saline group than in N-desulfated heparin group, demonstrating that N-desulfated heparin can significantly inhibit the bFGF expression of cancer cells. In vitro, the bFGF expression of human gastric cells was significantly increased with the extension of time. Treated with 0.1 or 1 mg/mL N-desulfated heparin for 12 and 24 h, bFGF expression was decreased significantly. Moreover, in each of N-desulfated heparin groups, bFGF mRNA expression was decreased compared with control group. bFGF mRNA expression was lower in 1 mg/mL N-desulfated heparin group than 0.1 mg/mL N-desulfated heparin group. In the same N-desulfated heparin concentration, the expression of bFGF mRNA at 24 h was lower than that at 12 h. Therefore, the inhibition of N-desulfated heparin on bFGF mRNA expression of human gastric carcinoma cells had dose- and time-dependent effects. The results suggest that N-desulfated heparin inhibits tumor angiogenesis by inhibiting expression of bFGF.

Sartippour et al. [29] concluded that nipple fluid bFGF levels were progressively elevated in high-risk and cancerous breasts compared with benign breasts. Barclay et al. [30] found that overexpression of bFGF mRNA by comparison with tumors underexpressing bFGF was associated with significantly increased risk for tumor recurrence. Hatziapostolou et al. [31] has showed that bFGF is a pleiotropic growth factor that has been implicated in prostate cancer formation and progression. According to the study, they found that exogenous bFGF significantly increased human prostate cancer LNCaP cell proliferation and migration. Heparin affin regulatory peptide (HARP) or pleiotrophin seems to be an important mediator of bFGF stimulatory effects. bFGF, through FGF receptors (FGFRs), significantly induced HARP expression and secretion by LNCaP cells and increased luciferase activity of a reporter gene vector carrying the full-length promoter of HARP gene. Activation of FGFR by bFGF in LNCaP cells leads to NAD(P)H oxidase-dependent hydrogen peroxide production, phosphorylation of ERK1/2 and p38, activation of AP-1, increased expression and secretion of HARP, and, finally, increased cell proliferation and migration. Pardo et al. [32] demonstrated that bFGF increased the expression of antiapoptotic proteins, XIAP, and Bcl-X(L) and triggered chemoresistance in SCLC cells. They found that these effects were mediated through the formation of a specific multiprotein complex comprising B-Raf, PKCepsilon, and S6K2. In a tetracycline-inducible system, increased S6K2 kinase activity triggers upregulation of XIAP, Bcl-X(L) and prosurvival effects. Zhao et al. [33] has reported that the measurement of plasma levels of such angiogenic factors as VEGF, bFGF, and MMP-9 in advanced NSCLC is helpful for prediction of metastasis tendency and evaluation of prognosis. Expression of the mRNAs of VEGF, flt-1, flk-1, and flg-1 (a receptor for bFGF) was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization (ISH) with cRNA probes. VEGF, bFGF, flt-1, and flk-1 were immunohistochemically detected in the neoplastic cells in HSAs; the staining intensity was stronger in HSAs than in hemangiomas [34].

In the present study, hemorrhage was never observed in N-desulfated heparin treated mice, suggesting that N-desulfated heparin has no obvious anticoagulant activity. In conclusion, bFGF produced by cancer cells is an angiogenic factor in human cancer tissue and plays an important role in tumor metastasis. N-desulfated heparin inhibits tumor metastasis by inhibiting expression of bFGF and angiogenesis. N-desulfated heparin can be used in the treatment of tumor metastasis.

Conflict of Interests

The authors state that there is no conflict of interests.

Acknowledgment

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Clinical Study

Ki-67 Antigen Overexpression Is Associated with the Metaplasia-Adenocarcinoma Sequence in Barrett's Esophagus

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Introduction. The objective of this study was to evaluate Ki-67 antigen expression in patients with Barrett's esophagus and esophageal adenocarcinoma and to assess its correlation with the metaplasia-esophageal adenocarcinoma progression. *Methods*. Using immunohistochemistry we evaluated the Ki-67 index in patients with Barrett's esophagus, esophageal adenocarcinoma, and controls. We included patients with endoscopically visible columnar mucosa of the distal esophagus (whose biopsies revealed specialized intestinal-type metaplasia), patients with esophageal and esophagogastric tumors types I and II, and patients with histologically normal gastric mucosa (control). *Results*. In the 57 patients studied there were no statistically significant differences between the groups with respect to age or race. Patients with cancer were predominantly men. The Ki-67 index averaged $10 \pm 4\%$ in patients with normal gastric mucosa ($n = 17$), $21 \pm 15\%$ in patients with Barrett's esophagus ($n = 21$), and $38 \pm 16\%$ in patients with cancer ($n = 19$). Ki-67 expression was significantly different between all groups ($P < 0.05$). There was a strong linear correlation between Ki-67 expression and the metaplasia-adenocarcinoma sequence ($P < 0.01$). In patients with cancer, Ki-67 was not associated with clinical or surgical staging. *Conclusions*. Ki-67 antigen has increased expression along the metaplasia-adenocarcinoma sequence. There is a strong linear correlation between Ki-67 proliferative activity and Barrett's carcinogenesis.

1. Introduction

Described 50 years ago by Norman Barrett, Barrett's esophagus (BE) is currently defined as an endoscopically visible columnar mucosa in the distal esophagus, of any extension, proved to harbor intestinal metaplasia on biopsy, highlighted by the presence of goblet cells [1]. Barrett's esophagus is a common disease, occurring in 10% of patients with gastroesophageal reflux disease [2]. It occurs as a complication of long-standing gastroesophageal reflux and is an important risk factor for the development of esophageal adenocarcinoma (EAC) [3–5]. The risk of cancer in BE patients is 0.2–2.9% per year, about 30 to 125 times that of the general population [6, 7].

The incidence of EAC is rising in the western world. Its prevalence exceeds that of squamous cell carcinoma and EAC is the most common type of esophageal cancer in some populations [8, 9]. Few epidemiological studies on EAC have been carried out in Brazil. Research conducted between 1987 and 1996 showed that adenocarcinomas represented 15% (53/349) of esophageal and esophagogastric tumors [10]. Esophageal adenocarcinoma is a lethal disease and effective treatment is reliant on early diagnosis [11–13]. Therefore, patients with BE require a rational followup, in order to allow early identification of malignant transformation. Current prognostic evaluation is based on the presence of dysplasia during serial endoscopic examinations. This classification has limitations, however, and results in heterogeneous groups. Up to 40% of resected specimens from patients with BE and high-grade dysplasia (HGD) contain EAC [2, 14]. The natural evolution of patients with low-grade dysplasia (LGD) is uncertain, partly due to intra-

and interobserver diagnostic variability, sampling errors, and variable regression rates for nondysplastic epithelium [15–18]. The percentage of cases involving progression to HGD and cancer can be as high as 28% and 15%, respectively [19].

Following evidence of dysplasia many patients will undergo excessive evaluations. However, some are only diagnosed with cancer at a late stage, in which there is already lymphatic spread; this results in poor outcomes [20, 21]. Prognostic molecular markers are thus sought to identify those patients at risk of developing cancer [19]. Barrett's carcinogenesis underlies a series of genetic and epigenetic events, revealed phenotypically as a sequence: metaplasia-dysplasia-adenocarcinoma [6, 22–28]. Among other mechanisms, uncontrolled proliferative activity takes place, independent of stimulatory and inhibitory control [29–32]. This proliferative activity has received a great deal of attention and has been studied using several techniques: tritiated thymidine incorporation into DNA, nuclear antigen detection (such as the Ki-67 and proliferating cell nuclear antigen (PCNA)), and expression of ornithine decarboxylase. Ki-67 has become the marker of choice due to its accuracy and easy feasibility. Its function is unknown and it is expressed within the nucleus of G1, S, G2, and M cells, but not G0 cells [33, 34].

Numerous studies have suggested that there is elevated Ki-67 expression in the metaplasia-dysplasia-adenocarcinoma sequence in BE [35–38]. Also, progressive Ki-67 expression has been described through the sequence between normal mucosa and dysplastic tissue or esophageal squamous cell carcinoma [39]. However, there is variable Ki-67 expression in EAC and inconclusive results along the metaplasia-dysplasia-adenocarcinoma sequence in BE. This study aimed to evaluate Ki-67 expression in patients with BE and EAC and to assess the correlation of this marker with the metaplasia-adenocarcinoma sequence.

2. Materials and Methods

2.1. Patients. The study population consisted of patients between the ages of 16 and 90 who were diagnosed with BE and EAC between August 2002 and December 2005. They were diagnosed and treated by the Surgery of the Esophagus, Stomach and Small Intestine Group at the Hospital de Clínicas de Porto Alegre (HCPA), Brazil.

We first reviewed anatomopathological records from the HCPA Pathology Service. The inclusion criteria were the following: (1) patients with dyspeptic symptoms and normal gastric mucosa on biopsy; (2) patients with esophageal columnar mucosa on endoscopy and intestinal-type metaplasia with goblet cells on biopsy; (3) patients with a diagnosis of EAC and esophagogastric junction tumor types I and II [40]. The exclusion criteria were (1) patients with intestinal metaplasia on biopsy without endoscopically visible columnar mucosa-cardiac intestinal metaplasia (CIM); (2) cases with insufficient material; (3) previous oncological treatment; (4) esophagogastric junction tumor type III; (5) BE patients who had received prior anti-reflux surgery; (6) previous history of carcinoma of other sites.

Taking all the clinical and histopathological data into account, the patients were divided into three groups: group 1 (controls), group 2 (BE), and group 3 (cancer). Sample size was calculated and it was found that at least 34 patients would be necessary: 12 in groups 1 and 2, respectively, and 10 for group 3. For nonparametric distribution, a sample size that is approximately 10% greater would be necessary (i.e., 38 patients).

2.2. Diagnostic Criteria for BE, Dysplasia, and Adenocarcinoma. The anatomopathological study was carried out separately by two experienced pathologists. Intestinal metaplasia was defined by the presence of goblet cells in the glandular mucosa. Dysplasia was defined as the presence of variation in nuclear size and shape, nuclear or nucleolar enlargement, increased nuclear to cytoplasmic ratio, hyperchromatism, and abnormal mitosis. Dysplasia was classified into negative, undefined, LGD, and HGD, as previously described [41–43]. Adenocarcinoma was characterized by the presence of atypical glands beyond the basal membrane, invading the lamina propria and the submucosa. The patients with EAC were staged according to the TNM (UICC-2004) classification.

2.3. Immunohistochemistry. Immunohistochemistry was performed at the Research Center of HCPA. Paraffin-embedded tissue sections fixed in formalin were used. Epitope retrieval was heat-induced in citrate buffer. Monoclonal antibody MIB-5 (DakoCytomation, Denmark) against the Ki-67 antigen was diluted 1 : 50. The avidin-biotin immunoperoxidase method was employed for Ki-67 staining, as described previously [34–37].

A Ki-67 index was determined for each patient, that is, the percentage of stained cells as a fraction of the total cells (at least 500) in an esophageal or gastric crypt (Figure 1), as described previously [36]. Positive nuclei stained brown (Figure 2). A "hot-spot" area was chosen for each patient. The Ki-67 index was calculated separately by two pathologists experienced in immunohistochemistry and blinded to the clinical information. The final Ki-67 index was the average of two measures for each patient.

2.4. Statistical Analyses. The Ki-67 index had parametric distribution and the data are presented as the mean ± standard deviation. Comparisons between continuous variables of the three groups were assessed using analysis of variance (ANOVA). The Tukey test was used to localize differences, when they were present. The linear correlation between variables was analyzed with the Pearson correlation coefficient. Comparisons between categorical variables were made using the Chi-square test. Statistical significance was assumed at $P < 0.05$. The software used was the Statistical Package for the Social Sciences (SPSS) version 12.0.

2.5. Ethics. This study was evaluated and approved by the Group of Research and Post-Graduation and Bioethics Committee of the HCPA, following all recommended ethical norms. The paraffin-embedded tissue specimens were

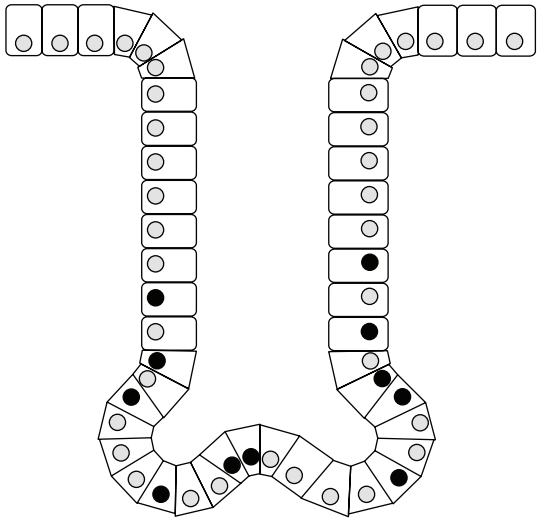

Figure 1: Ki-67 index: esophageal crypt scheme. Ki-67 index = ●/● + ○ × 100%, where closed circles represent marked nuclei. (Adapted from [44]).

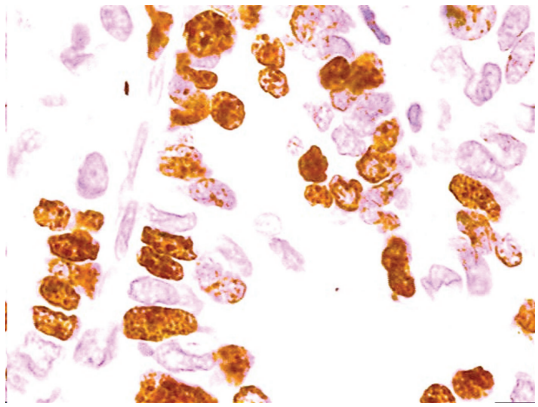

Figure 2: Example of Ki-67 immunohistochemical staining of esophageal tissue in a patient with esophageal adenocarcinoma, in 400x field (stained cells are marked with an arrow).

obtained from the HCPA Pathology Service's archives. Patients did not participate directly in the study, and their treatment protocols were not modified by the research. The clinical data, collected from the medical records, was used confidentially and anonymously.

3. Results

Initially 80 patients were selected, of which 23 were excluded: 6 cases of CIM, 5 subcardial adenocarcinomas (Type III), and 12 which provided insufficient material. Of the remaining 57 patients, 19 had esophageal or esophagogastric adenocarcinoma, 21 had BE, and 17 were controls. The demographic data are presented in Table 1. There was no difference between the groups with respect to age and race. Men predominated in group 3 (cancer).

The average overall Ki-67 index was 23.62 ± 17.6%. The average Ki-67 was 10.29±4.6% in the controls, 21.26± 15.1% in the BE patients, and 38 ± 16% in the EAC patients (Figures 3 and 4). Ki-67 increased through groups 1 to 3, and there was a significant difference in the Ki-67 index among all three groups (Figure 5). There was a strong linear correlation ($r = 0.6$) between Ki-67 and the progression from control to metaplasia to adenocarcinoma (Figure 6) ($P < 0.01$). No significant interobserver variability was found.

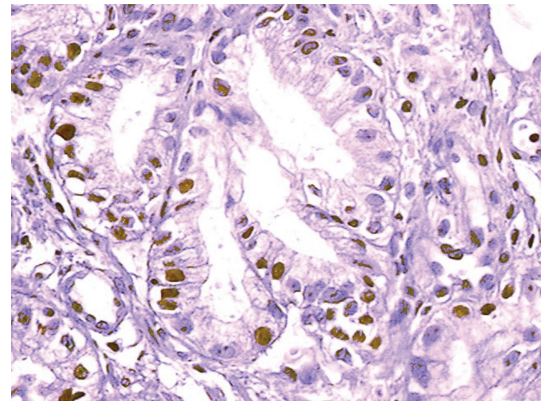

Figure 3: Example of immunohistochemical staining for Ki-67 antigen in Barrett's esophagus under 200x microscopic magnification (stained cells are marked with an arrow).

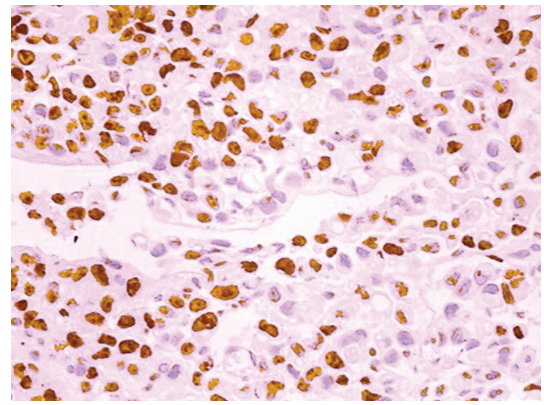

Figure 4: Example of immunohistochemical staining for Ki-67 antigen in esophageal adenocarcinoma under 200x microscopic magnification (stained cells are marked with an arrow).

The columnar epithelium extension in patients with BE was 5.29 ± 3.39 cm. Short-segment BE (<3 cm) was found in 23.8% of the patients, while long-segment BE (>3 cm) was found in 76.2%. There was no correlation between columnar mucosa extension and the Ki-67 index. Three (15%) patients with BE had LGD, whose average Ki-67 index was 17.5 ± 13.2%. Considering the small sample size of patients with dysplasia, we did not analyze this group. Ninety percent of the patients with BE had hiatal hernia, which averaged 2.95 ± 1.9 cm. The size of the hiatal hernia did not correlate with Ki-67 expression.

Patients with EAC were classified according to stage. Stage 1 occurred in 5.9% of patients while stages 2, 3, and 4 corresponded to 31.6% of cases, respectively. No statistical difference in the Ki-67 index was observed between these

Table 1: Demographic data from patients whose tissue was used to investigate the relationship between expression of the antigen Ki-67 and stages within the metaplasia-adenocarcinoma sequence.

	Group 1 (control) $n = 17$	Group 2 (BE) $n = 21$	Group 3 (cancer) $n = 19$	Total $n = 57$	P value
Age (mean ± SD)	55.7 ± 12.1	52.52 ± 20.28	62.89 ± 13.54	56.26 ± 16.48	$P = 0.082$
Gender (%)					
Men	8 (47)	9 (42.9)	15 (78.9)	32 (56.1)	$P = 0.048$*
Women	9 (52.9)	12 (57.1)	4 (21.1)	25 (43.9)	
Race					
Caucasian	16 (94.1)	18 (85.7)	19 (100)	53 (93)	$P = 0.20$
Black	1 (5.9)	3 (14.3)	0 (0)	4 (7)	

* Patients with cancer were more likely to be men.
BE: Barrett's esophagus.

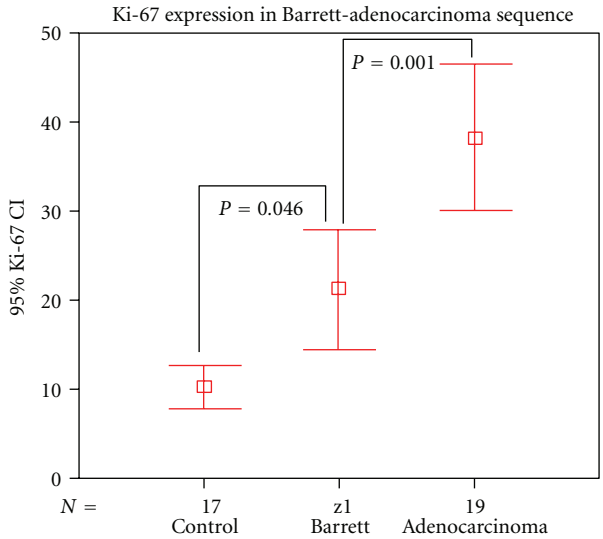

Figure 5: Ki-67 index variation between the three different groups (control, Barrett's esophagus, and adenocarcinoma). There is increased expression of Ki-67 along the Barrett-adenocarcinoma sequence.

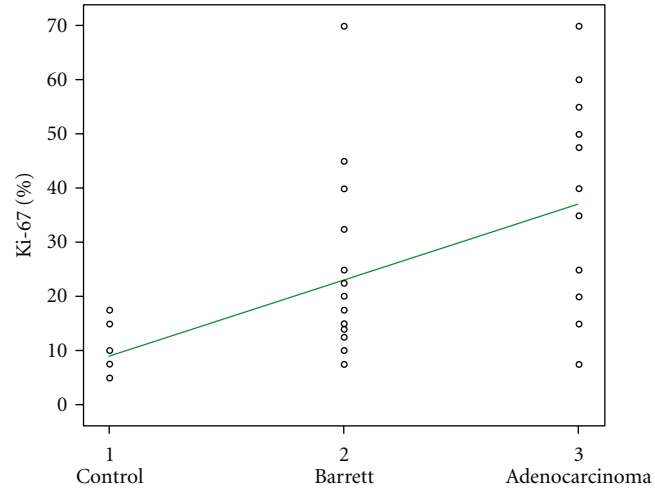

Figure 6: The correlation between Ki-67 antigen and the Barrett's esophagus to adenocarcinoma sequence. Pearson coefficient = 0.6 ($P < 0.01$).

stages. Eleven patients were resected, with a curative intent for five of these and a palliative intent for six. In eight patients, surgery was not carried out due to advanced disease ($n = 6$) or prohibitive surgical risk ($n = 2$). In the majority of cases, the tumor was moderately differentiated (70%). There was no difference in Ki-67 index relative to tumor differentiation. In patients who received surgery, more than 80% had muscularis propria invasion or deeper and more than 50% had regional node metastasis. There was no association between the Ki-67 index and tumor (T) and node (N) stages, respectively.

4. Discussion

As Barrett's carcinogenesis is a multistep process that follows the typical metaplasia-dysplasia-adenocarcinoma sequence, prognostic markers for disease progression have been sought. These have included factors within the cell cycle, oncogenes, and tumor suppressor genes. Increased proliferative activity has been reported in different tumors [45–47]. Currently, Ki-67 is one of the most studied markers of cell proliferation. Increased Ki-67 expression has previously been demonstrated in 165 digestive carcinomas: gastric, esophageal, colonic, and rectal [48]. Ki-67 expression has also been described in esophageal squamous cell carcinoma [39].

Initial studies assessing Ki-67 antigen used flow cytometry. The results obtained using this method did not show significant differences in Ki-67 expression, when comparing BE patients with different degrees of dysplasia and adenocarcinoma [49, 50]. The disadvantages of flow cytometry include the requirement for frozen sections and sophisticated equipment, tissue architecture compromise, and its laborintensiveness [51–53].

The Ki-67 index (percentage of stained cells/total cells) has been used to evaluate the proliferative activity of tumors and requires immunohistochemistry in paraffin-embedded tissue. The Ki-67 index is now the method of choice for proliferation studies, due to its accuracy and ease of use. Although electronic counting is sometimes used for this method, we did not have access to the necessary equipment

and used the more conventional manual counting method [33, 34, 41, 47, 54]. This was done by two experienced pathologists, who were blinded from the clinical data, to minimize bias.

Hong et al. used the gastric epithelium as a control to evaluate Ki-67 in BE [55]. We chose to use the same control, as the histological architecture and cellularity of the gastric mucosa closely resembles BE, with its mucous glands and crypts, which represent the primary proliferative zone. We considered the stratified squamous epithelium to be unsuitable as a control. It has a different architecture, is devoid of glands, and proliferative activity is restricted to its basal layer. Gastric mucosa proliferative behavior also shows a greater correlation with that of BE [49].

The crypt stratification, according to its depth, has been evaluated in some studies. These studies showed a difference in Ki-67 distribution, mainly between LGD and HGD. The proliferative activity moved from the deep compartment of crypt, in the BE with LGD, to the superficial compartment, in the BE with HGD [38, 55]. We did not stratify the epithelium, as we considered it subjective, since histological sections irregularly divide crypts in different depths and directions. In patients with BE we counted Ki-67 throughout the entire crypt, in the "hot-spot" area, and counted at least 500 cells per patient. Although stratification has been shown to be important (particularly in the differentiation of patients with LGD and HGD) we had few patients with dysplasia, they were not analyzed as a group, and epithelium stratification was unnecessary.

Polkowski et al. analyzed the Ki-67 index in 25 esophagectomy-resected specimens, in different histological areas of BE [56]. In the "hot-spot," the Ki-67 index averaged 45% in areas without dysplasia, 45% in undefined areas of dysplasia, 46% in LGD, and 55% in HGD. Despite the small differences, there was a significant linear correlation between Ki-67 and the observed histological progression. The size of the proliferative zone also increased significantly with disease progression [56]. Despite using esophagectomy specimens, that study did not report Ki-67 positivity in cancerous areas. Furthermore, a control group was not used.

Lauwers et al. analyzed Ki-67 expression in 20 esophagectomy specimens and reported 10% positivity in BE without dysplasia, 20% in LGD, and 50% in HGD [38]. The expression of Ki-67 in cancer areas was not reported.

Hong et al. evaluated the Ki-67 index in 43 patients with BE [55]. Ki-67 expression was 13% in the gastric mucosa (control), 33% in BE without dysplasia, 40% in BE with LGD, and 33% in HGD. There were only 5 cases of esophageal adenocarcinoma and Ki-67 expression was 38% [55]. When stratifying the glands those authors showed a significant difference in Ki-67 expression between the groups and reported a superficial proliferating zone in HGD patients.

Rioux-Leclercq et al. assessed Ki-67 expression in 44 esophagectomy specimens, in different histological areas [37]. Areas with BE and LGD were positive for Ki-67 in 14% of patients, EB and HGD in 73%, and EAC in 87%. Significant increase in the prevalence of Ki-67 in the sequence of dysplasia to adenocarcinoma was found [37]. However, Ki-67 in BE without dysplasia and the control group was not reported. Moreover, this study considered Ki-67 to be positive when the index was 10% or more. Such a criterion is somewhat arbitrary since it has not been used in any other publications.

In a recent study, conducted by Feith et al., different histological areas were evaluated for Ki-67 in 24 esophagectomy specimens [36]. Ki-67 expression increased significantly in the following sequence: squamous mucosa (20%), BE without dysplasia (35%), BE with dysplasia (45%), and adenocarcinoma (60%) [36].

Another recent study by Szachnowicz et al. evaluated Ki-67 in 13 esophagectomy specimens and demonstrated "moderate" or "strong" proliferative activity in all cases of BE ($n = 9$) and EAC ($n = 12$) [11]. They did not determine the Ki-67 index, but described the staining of Ki-67 in four degrees ("absent," "weak," "moderate," and "strong") [11]. This criterion has, however, not been used before, making comparisons impossible. Statistical analysis was not done for Ki-67 expression.

Bhargava et al. conducted a prospective study on the behavior of different markers in BE, using a rigorous esophageal biopsy protocol [57]. Ki-67 was evaluated in only the six initial patients, with a total of 200 biopsy specimens. A significant association of Ki-67 with the presence of dysplasia was observed, even in this small group of patients. Ki-67 was positive in 8 of 10 (80%) specimens with dysplasia and absent in 179 of 181 (99%) specimens without dysplasia [57]. However, the method used to assess Ki-67 was not clear, and those authors report it as a qualitative variable. Moreover, despite the high number of biopsy samples, the number of patients is reduced, with limited sample representation, as 30% (2/6) of the patients presented dysplasia.

In summary, we found inconclusive and heterogeneous results in all of these previous studies, although there is agreement on a correlation between Ki-67 and disease evolution. In our study, the average Ki-67 expression was 10% in the normal gastric mucosa, in accordance with the literature. Patients with BE and EAC showed a Ki-67 positivity of 21% and 38%, respectively. These results differ from those previously reported, that is, up to 45% positivity in BE and 60% in cancer. These differences may be partly due to small and unrepresentative sample sizes, taken from studies based on different histological areas of esophagectomy-resected specimens. In such studies, only one patient may be analyzed using several histological sections. Our study sample is patient based and not specimen based, and each patient has only one diagnosis. Variations in immunohistochemical technique may also partly explain the variable results. These may include the types of antibodies, antigenic presentation, and assessment of the marker.

We demonstrated a significant correlation between the Ki-67 index, indicating proliferative activity, and the Barrett's esophagus to adenocarcinoma progression. The results are concordant with the literature and confirm the progressive nature of this disease relative to the increasing prevalence of this marker.

In patients with EAC, we did not find an association between Ki-67 expression and either clinical staging, tumor penetration or nodal spread. These results suggest a limited

role for Ki-67 as a prognostic marker in patients with this cancer; however, the small sample size used to carry out comparisons within the group must be considered. Other studies have similarly not found significant differences in Ki-67 expression relative to cancer staging [36].

In the last 10 years, more than 10 studies have noted that adequate gastroesophageal reflux control is associated with the histological regression of Barrett's esophagus. Antireflux surgery was shown to be an important predictive factor for histological regression, occurring in 36% of patients undergoing surgery [18]. Identification of this subgroup of patients prone to regression is an appropriate field for future research, where molecular markers may contribute to treatment decisions for each patient.

Regarding methodological aspects, we would like to point some fragilities of this study. First, as patients with cancer were more likely to be men, we could not rule out the impact of smoking or alcohol consumption. Also, the sample of patients is small; however we were able to reach statistical significance. At last, as this is a retrospective study, we could not obtain normal esophageal mucosa biopsy.

We found small absolute differences in Ki-67 expression between the three groups (controls, BE, and EAC), despite the fact that differences were statistically significant. This suggests a limited role for Ki-67 as a powerful marker of Barrett's carcinogenesis. To better evaluate the prognostic value of this marker in the metaplasia-dysplasia-adenocarcinoma sequence, a prospective study with followup of patients at risk should ideally be conducted. Considering the variability of published results in this field, future studies require better standardization of the methods to allow improved comparisons between the outcomes.

5. Conclusions

The Ki-67 index was 10% in patients with normal gastric mucosa (control), 21% in patients with BE, and 38% in EAC patients. There was a significant difference between all the groups, with an increasing expression of Ki-67 relative to the progression of BE to adenocarcinoma.

There was linear correlation between Ki-67 expression and the metaplasia-adenocarcinoma progression in BE, demonstrating an increasing Ki-67 positivity relative to disease evolution.

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Clinical Study

miR-21 Is a Promising Novel Biomarker for Lymph Node Metastasis in Patients with Gastric Cancer

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Background. Gastric cancer (GC) is an important malignant disease around the world. Abnormalities of microRNAs (miRNAs) have been implicated in carcinogenesis of various cancers. In the present study, we examined miR-21 expression in human gastric cancer with lymph node metastasis and attempted to uncover its relationship with clinicopathologic data, especially with lymph node metastasis. *Materials and Methods*. The expression levels of miR-21 in the tumor specimens of GC patients were quantified by RT-PCR. The correlation between miR-21 level and multiple clinicopathological factors was then examined by Mann-Whitney test, Kaplan-Meier survival analysis, and operating characteristic (ROC) analysis. *Results*. The expression level of miR-21 was higher in GC patients with lymph node metastasis than in those without lymph node metastasis ($P < 0.05$). Expression level of miR-21 was significantly correlated with histologic type, T stage, lymph node metastasis and pTNM stage. The overall survival rates in GC patients with low upregulated miR-21 expression were significantly higher than those with high upregulated miR-21 ($P < 0.05$). *Conclusion*. A close association is implicated between the elevated miR-21and lymph node metastasis, which could potentially be exploited as a practical biomarker for lymph node metastasis in patients with GC.

1. Introduction

Gastric cancer (GC) is the fourth most prevalent malignant cancer worldwide and is the second most frequent cause of cancer death [1]. Despite many advances made in GC therapy, the prognosis for patients with GC remains unsatisfying. Because of early detection in screening programmes in Japan, survival is good (52%), whereas survival in the USA, Europe, and China generally is only 20–25% for the delayed diagnosis [2]. Five-year survival rate for advanced or metastatic gastric cancer is nearly 5–20%, with median overall survival being less than 1 year [3, 4]. Therefore, it is necessary to find prognostic markers that could accurately indicate biological characteristics of GC and supply the evidence for early diagnosis and predicting the clinical outcome so as to improve the clinical management of GC patients. With the advances in diagnostic and operative technologies, surgical decisions have been made based on tumor stage [5, 6]. In order to tailor surgical therapy, it is necessary to assess clinical stage and depth of tumor invasion. However, the method for prediction of lymph node metastasis, another important determinant for prognosis, has not yet been well established.

MicroRNAs (miRNAs), a species of small noncoding RNA of about 21–23 nucleotides, could interact with their target mRNAs to interfere with the translation by promoting mRNA degradation or to block translation by binding to partial homology to target mRNA in the 39-untranslated region [7, 8]. It has been reported that miRNAs play important roles in various human biological processes such as metabolism, differentiation, cell proliferation, and apoptosis. Abnormalities of miRNA are suggested in carcinogenesis of various cancers, indicating that miRNAs could be used as molecular biomarkers for diagnosis of cancer and prediction of prognosis [9–12]. MiR-21 has been reported to be elevated in multiple human solid tumors including lung, breast, stomach, prostate, colon, and pancreatic cancer and their respective normal adjacent tissue. Meanwhile, serum miR-21 was also found to be overexpressed in many cancers such as

diffuse large B-cell lymphoma, ovarian cancer, prostate cancer, or breast cancer. Evidence supports the hypothesis that miR-21 is a central oncomiR [13–16].

In the present study, we explore miR-21 expression and its correlation with clinicopathological factors in gastric cancer. Furthermore, we also determine whether miR-21 expression in lymph node might be a molecular biomarker for predicting the lymph node metastasis of GC patients.

2. Materials and Methods

2.1. Patients and Samples. Tumor specimens and normal control tissues were collected from 86 patients who underwent surgical treatment for histologically proven gastric adenocarcinoma at the Department of Surgery, Nanjing Second People's Hospital, Shanghai Sixth People's Hospital, and Shanghai Putuo Hospital, from January 2006 to December 2008. None of the patients had been administered by chemotherapy or radiotherapy prior to undergoing surgical resection. Metastatic lymph nodes were also harvested during gastrectomy. Clinical stage of GC was assessed on the basis of the tumor node metastasis (TNM) classification system recommended by the International Union against Cancer. For accurate N staging, more than 15 lymph nodes in one patient were collected by means of a careful manual palpation. Demographic and clinicopathological details of patients were collected from electronic patient records. The study was approved by the Institutional Review Board of Nanjing Second People's Hospital, Shanghai Sixth People's Hospital, and Shanghai Putuo Hospital. *Written informed* consent was *obtained* from each participant.

2.2. RNA Extraction and qRT-PCR. Total RNA was extracted from frozen specimens using Trizol (Invitrogen) following the manufacturer's guide. Total RNA was eluted in 100 mL and stored at −20°C. 5 mL of RNA was used to measure the expression of miR-21 by quantitative RT-PCR (qRT-PCR) with the TaqManH miRNA reverse transcription kit and the TaqManH miRNA assay-specific RT primers for miR-21 according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA). The expression of Let-7a was used as internal control. Real-time PCR was performed with 3 mL of each cDNA on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) in duplicates. C_T value is defined as the number of PCR cycles at which the fluorescent signal crosses the threshold. The difference of C_T values between internal control and miR-21was presented as $-\Delta C_T$. $\Delta\Delta C_T$ is the difference of ΔC_T values between paired specimens. $2^{\Delta\Delta CT}$ represents the exponential value of ΔC_T, which means fold change in expression.

2.3. Statistical Analysis. The correlation between up-regulated miR-21 and different clinical factors was assessed by Mann-Whitney analysis, paired *t*-test linear regression, and Kaplan-Meier survival analysis. To determine to which extent the obtained $-\Delta C_T$ value of miR-21 could efficiently separate different clinical subsettings, operating characteristic (ROC) analysis was generated, and the sensitivity and specificity of the optimum cut-off point were defined as those values that maximized the area under the ROC curve (AUC). Data analysis was done using R software statistical environment (version 2.14.1; R Development Core Team, Vienna, Austria). All statistical tests were two-sided, and P value of less than .05 was considered statistically significant.

3. Results

3.1. Expression of miR-21 in GC Specimens. The expression level of miR-21 was analyzed by qRT-PCR. The expression level of internal control, Let-7a, showed no significant difference between the three groups (data not shown). We found significant difference between normal controls and patients without lymph node metastasis ($P < 0.05$). Furthermore, there is significant increase of miR-21 expression levels in patients with lymph node metastasis compared to normal controls and patients without lymph node metastasis (Figure 1(a)). A cut-off value of 5.12 was best distinguished in patients with lymph node metastasis and without lymph node metastasis, and the AUC value was 0.79 (Figure 1(b)).

3.2. Correlation of miR-21 Expression with Clinicopathological Factors of GC Patients. The expression of miR-21 was found to be highly upregulated in 30 (34.9%) of 86 cases, whereas the remaining 56 cases (65.1%) were classified as having low up-regulated expression. To investigate the clinical significance of up-regulated miR-21, the relationship between miR-21 and clinicopathological factors was further assessed. As shown in Table 1, high up-regulated miR-21 expression appeared to be significantly associated with more histologic type ($P < .001$), T stage ($P < .001$), lymph node metastasis ($P < .001$) and pTNM stage ($P < .05$) in univariate analysis. Multivariate analysis found that pTNM stage ($P = .001$) and lymph node metastasis ($P = .001$) were statistically significant.

3.3. Correlation of miR-21 Expression with Prognosis of GC Patients. The overall survival rates in the patients with or without lymph node metastasis and with low or high up-regulated expression of miR-21 were statistically estimated. As shown in Figure 2, the median overall survival time in patients with high and low up-regulated miR-21 expression levels was nearly 11.2 and 13.8 months, respectively. The survival difference between these two groups was statistically significant ($P < 0.05$). We found that there were no significant differences between the patients with lymph node metastasis and high up-regulated expression of miR-21 and without lymph node metastasis and low up-regulated expression of miR-21 ($P > 0.05$).

4. Discussion

To develop sensitive and specific minimally invasive molecular biomarkers for tailored management of cancers is a major challenge in clinical oncology. Dozens of studies have shown that miRNAs might be used as potential molecular biomarkers for human malignancies. Although numerous

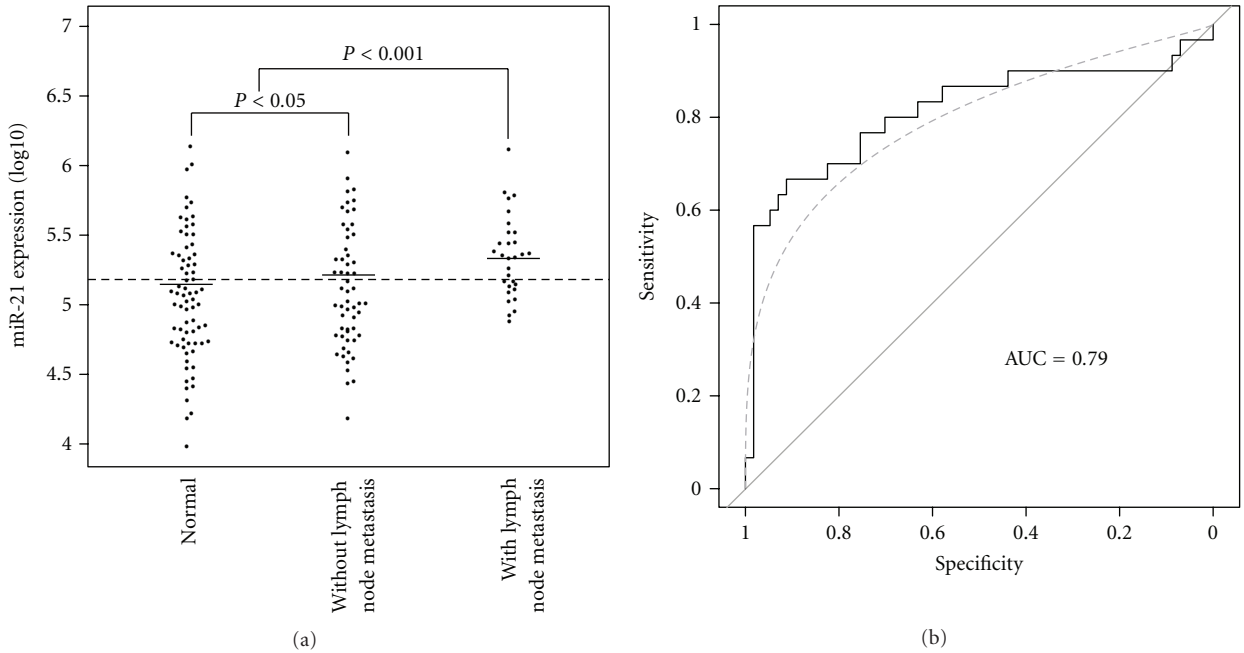

Figure 1: Comparison of levels of miR-21 in samples from gastric cancer (GC) patients without lymph node metastasis ($n = 56$), GC patients with lymph node metastasis ($n = 30$), and normal controls ($n = 72$). The lines denote the medians. Dot lines at the y-axis denote cut-off values. A significant difference between all histological groups was examined by ANOVA test. NS denotes no significant difference between groups. Receiver operating characteristics (ROCs) curves were used to discriminate patients with and without lymph node metastasis.

studies have been published for investigating the effect of the abnormal expression levels of miRNAs on the tumorigenesis, there is only a paucity of reports dealing with clinical impact of miRNAs in patients with lymph node metastasis [17–21]. Therefore, identification of miRNAs expression in patients with lymph node metastasis will be helpful to facilitate the clinical management of GC.

In this study, we have established the potentiality of miR-21 in the tumor tissue as a biomarker for lymph node metastasis. Using qRT-PCR, miR-21 was identified to be highly upregulated in GC patients with lymph node metastasis compared to patients with no lymph node metastasis ($P < 0.05$). ROC curve showed that the AUC value was nearly 0.8, which indicated that the expression level of miR-21 might be used to predict the lymph node metastasis in patients with GC. A previous study reported by Chan et al. also showed that miR-21 was overexpressed in most of the gastric cancer patients [22]. In addition, miR-21 and its precursor have been reported to be upregulated in many human malignancies, including pancreatic cancer, breast cancer, colorectal cancer, glioblastomas, and lung cancer [20, 23, 24].

The molecular mechanisms of regulating the expression of miR-21 in GC are rarely reported, especially the reports of evaluating the correlation between miR-21 expression level and clinical stage, lymph node metastasis, and prognosis of GC patients. The evidence was derived from the recently published reports. Suppression of miR-21 in MCF-7 cell, which overexpressed miR-21, could increase apoptosis and decrease cell proliferation, and knockdown of miR-21 in glioblastoma cells also showed that this miRNA has an anti-apoptotic function [25, 26]. Furthermore, two direct targets of miR-21, PDCD4 and maspin, which could decrease the metastasis of malignancies, have been found [27]. Taken other line of evidence together, a possible role of miR-21 as an oncogene has been hypothesized, including proliferation, cell cycle, metastasis, and chemosensitivity of tumor cells by targeting several tumor suppressor genes such as PTEN, MARCKS, PDCD4, and Cdc25A [28–31]. However, additional studies should be designed to investigate the molecular mechanisms of both the cause and effect of altered expression of miR-21 in GC.

Our present study next focused on the potential relationship between the expression level of up-regulated miR-21 and various GC clinicopathological factors, as well as the prognosis of the patients. It is worth noting that high up-regulated expression level of miR-21 was significantly correlated with histologic type, T stage, lymph node metastasis, pTNM stage, and poor overall survival of the patients with GC. High up-regulated expression of miR-21 in GCs with lymph node metastases indicates that its up-regulation was acquired in the course of tumor progression and, in particular, during the acquisition of metastatic potential. These results suggest that miR-21 could serve as a prognostic marker for prognosis of GC patients. However, the reports published by chan et al. did not show that higher expression of miR-21 affected the clinical prognosis of gastric cancer patients [22]. A potential important reason is that the patient cohort is too small to find the statistically significant difference, which only included 24 and 13 patients in group

Table 1: Relationship between clinicopathological factors and miR-21 expression in patients with GC.

Parameters	miR-21 expression		P value*
	Low ($n = 56$)	High ($n = 30$)	
Age (means ± SD, years)	56.03 ± 12.86	56.26 ± 11.14	0.436
Gender			0.128
Male	35 (62.3%)	20 (66.1%)	
Female	21 (37.7%)	10 (33.9%)	
Tumor location			0.526
Proximal	3 (5%)	1 (3%)	
Body	24 (42.6%)	12 (41%)	
Distal	28 (50.4%)	17 (56%)	
Histologic type			<0.001
Differentiated	19 (34.3%)	7 (22.5%)	
Undifferentiated	37 (65.7%)	23 (77.5%)	
T stage			<0.001
T1	37 (66.7%)	15 (49.3%)	
T2	12 (21.4%)	7 (23.6%)	
T3	7 (11.9%)	5 (18.1%)	
T4	0 (0%)	3 (9%)	
Lymph node metastasis			<0.001
Present	17 (30.5%)	26 (85.3%)	
Absent	39 (69.5%)	4 (14.7%)	
Liver metastasis			0.266
Present	27 (47.8%)	15 (50.3%)	
Absent	29 (52.2%)	15 (49.7%)	
Peritoneal metastasis			0.137
Present	32 (57.8%)	18 (61.2%)	
Absent	24 (42.2%)	12 (38.8%)	
pTNM stage			<0.05
I	29 (52.6%)	12 (41.1%)	
II	16 (27.8%)	7 (21.8%)	
III	6 (11.3%)	6 (19.3%)	
IV	5 (8.3%)	5 (17.8%)	

*P value was tested in univariate analysis.

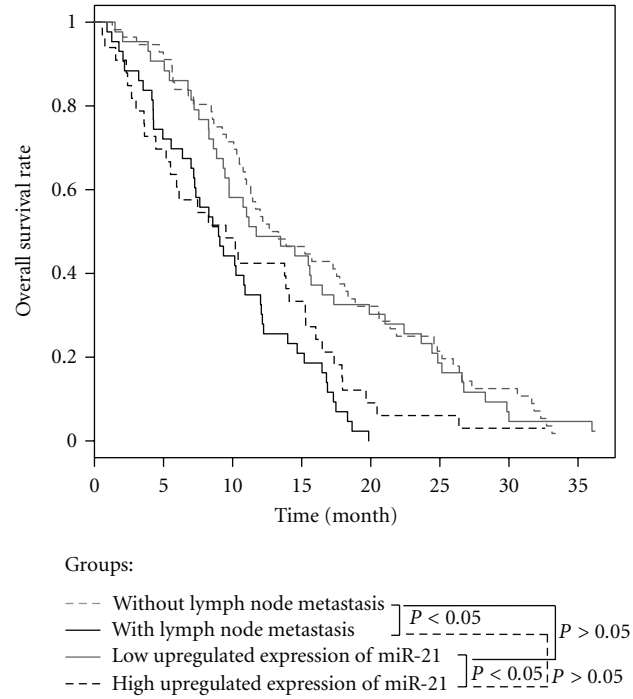

Figure 2: Kaplan-Meier survival curves of GC patients. The overall survival rate in patients with high miR-21 expression was significantly lower than that in those with low miR-21 expression ($P < 0.05$).

of <2-fold and ⩾2-fold elevated expression, respectively. Meanwhile, the design of our study was also different from the report by chan et al. In our study, we mainly examined the miR-21 expression in GC with lymph node metastasis, 56 and 30 GC patients without and with lymph node metastasis were included in the present study, respectively, and in the study reported by chan et al., only 2 and 35 GC patients without and with lymph node metastasis were included, respectively. Other researchers also reported the clinical significance of miR-21expression in human cancers. Yan et al. reported that miR-21 overexpression was correlated with specific breast cancer clinicopathological features, advanced tumor stage, lymph node metastasis, and poor survival of the patients [20]. Other reports showed that miR-21 expression in lung cancer was significantly correlated with advanced TNM stage, presence of lymph node metastasis, and overall survival [24]. This result indicates that miR-21 expression level could be used for the prediction of the clinical outcome. Of course, further prospective studies with a larger cohort are needed to confirm its prognostic significance in GC patients.

In conclusion, high up-regulated expression of miR-21 in GC was correlated with lymph node metastasis. Furthermore, the expression level of miR-21 might be also a potential prognostic factor for GC patients. Further studies are needed to verify the impact of miR-21 expression on gastric cancer, including the metastasis and prognosis.

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Research Article

Epidermal Growth Factor Receptor Expression in Esophageal Adenocarcinoma: Relationship with Tumor Stage and Survival after Esophagectomy

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Background and Aims. Esophageal adenocarcinoma (EA) is an aggressive tumor with increasing incidence in occidental countries. Several prognostic biomarkers have been proposed, including epidermal growth factor receptor (EGFR). The aim of this study was to assess whether EGFR expression predicts EA staging and patient survival. *Methods*. In this historical cohort, consecutive patients with EA managed between 2000 and 2010 were considered eligible for the study. Surgical specimens of patients treated with transhiatal esophagectomy were evaluated to establish EGFR expression and tumor differentiation. Staging was classified according with tumor-node-metastasis (TNM) system. Survival was determined according to either medical register or patient's family contact. *Results*. Thirty-seven patients who underwent esophagectomy without presurgical chemotherapy or radiotherapy were studied. EGFR expression was found in 16 patients (43%). EGFR expression was more frequent as higher was the TNM (I and II = 0% versus III = 47% versus IV = 100%; $P < 0.001$). Average survival in months was significantly shorter in the group of patients with EGFR expression (10.5 versus 21.7; $P = 0.001$). *Conclusions*. In patients with esophageal adenocarcinoma treated with transhiatal esophagectomy, EGFR expression was related to higher TNM staging and shorter survival. EGFR expression might be assumed as a prognostic marker for esophageal adenocarcinoma.

1. Introduction

Esophageal adenocarcinoma (EA) is an aggressive tumor with increasing incidence in several countries [1–5]. Optimistic five-year survival reaches 25% in patients treated with esophagectomy [6]. Adverse biological behavior and late diagnosis explain at least in part the poor prognosis of EA [7], pointing to the need for new strategies to improve patient selection and outcome prediction.

Gastroesophageal reflux disease (GERD) is a well-known risk factor for EA, particularly in the presence of Barrett's esophagus. This condition increases the likelihood of EA 30 times [8], with incidence of 1 new case of EA in 200 patients per year [9]. Studies also suggest a higher risk for patients with long-segment Barrett's esophagus and a greater risk in men compared with women [1, 10]. Other established risk factors for EA include obesity and smoking [11–13].

Among prognostic tools, tumor staging using TNM system is widely employed in the management of patients with EA [14]. Staging is performed by imaging studies, but in many cases a laparoscopic or thoracoscopic intervention is necessary [15]. Prior studies have introduced biomarkers

to predict the prognosis of EA. Mutation in p53 gene was first described as a marker of poor prognosis, regardless of TNM status [16]. More recently, epidermal growth factor receptor (EGFR) has received attention by its prognostic capability giving its participation in the control of epithelial cell multiplication. However, EGFR may be overexpressed in esophageal cancer, either in adenocarcinoma or squamous cell carcinoma [17–19].

It has been demonstrated that EGFR overexpression may be related with higher pathological TNM (pTNM) staging and poor cellular differentiation in EA patients [18–20]. Furthermore, EGFR has been linked with metastasis and decreased survival in these patients [18–20]. However, such prognostic studies included different surgical approaches for treatment of adenocarcinoma.

The hypothesis of the present study is that EGFR might be a prognostic marker for patients with EA treated with transhiatal esophagectomy, a widely accepted surgical technique [21]. Therefore, the aim of this study was to assess whether EGFR expression predicts tumor staging and survival in EA patients treated with a standardized surgical technique.

2. Methods

2.1. Patients. In this retrospective cohort, we reviewed all cases of EA managed at Hospital de Clínicas de Porto Alegre (HCPA) between January 2000 and December 2010. Patients were selected if they met the following criteria: (1) adenocarcinoma located in the esophagus or gastroesophageal junction (Siewert I and II); (2) treatment with transhiatal esophagectomy. Patients were excluded according to the following criteria: (1) neoadjuvant treatment with radiotherapy or chemotherapy; and (2) missing of pathology or follow-up data; (3) nonsurgical treatment; (4) Siewert III tumor. Data regarding survival were collected from medical registers or phone contact with patient's family.

This study was conducted according to the rules of the Brazilian Ethics and approved by the Ethical Committee of the HCPA (CONEP 198984/GPPG HCPA 08-300).

2.2. Transhiatal Esophagectomy. Patients were operated following a standardized surgical approach carried out by the same surgical team. Transhiatal esophagectomy was performed as described elsewhere [22]. Briefly, patients underwent laparotomy and cervicotomy, followed by diaphragm hiatus opening and esophageal dissection with periesophageal lymphadenectomy. The esophagus was sectioned proximally in the cervical segment and distally combined with proximal gastrectomy. Alimentary transit was reconstructed with anastomosis between gastric tube and cervical esophagus.

2.3. Immunohistochemistry Analysis. Determination of EGFR expression with immunohistochemistry was carried out following a published protocol [23]. Briefly, blocks with tumor tissue were first embedded in paraffin for posterior analysis of slices stained with hematoxylin and eosin. The slices were cut in 5 μm, followed by deparaffinization and rehydration in distilled water. They underwent antigen retrieval with Proteinase K (Dako) for 5 min and washed in distilled water. Subsequently they were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and further washed with distilled water for 5 min. The monoclonal anti-human EGFR, clone H11 (anti-EGFR, Dako) was applied to slices at a dilution of 1 : 200 and incubated for 60 min, rinsed in peroxidase blocking solution (PBS) and incubated with streptavidin (1 : 20 dilution) by 30 min at room temperature, and washed twice with PBS for 5 min. Thereafter, chromogen diaminobenzidine was applied for 5 min, washed in common water for 3 min, and then washed in distilled water. Finally, the slices were stained with hematoxylin for 2 min, dehydrated with alcohol, and mounted for analysis.

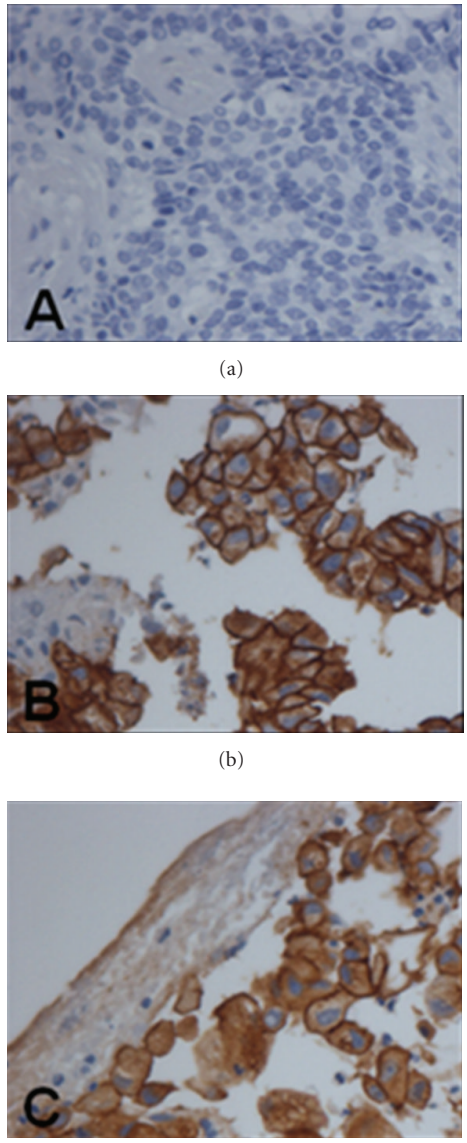
2.4. Analysis of EGFR Expression. EGFR expression was considered positive when membrane tumor cell was stained in brown color. An external positive control was performed with placenta tissue and a cell line of esophageal squamous carcinoma with positive EGFR (Figure 1).

Tissue analysis was performed by trained investigators and reviewed by an experienced pathologist blinded to clinical and pathological patient's information.

2.5. Statistical Analysis. Data are presented as mean ± SD, and frequencies and percentages when appropriate. The following variables were analyzed: gender, age, tumor place, tumor differentiation, surgical staging, and survival. These variables were related to EGFR expression (yes/no). Quantitative data were analyzed using t-test, whereas qualitative variables were tested with chi-square test. Survival was described using Kaplan-Meier analysis. The P value was considered statistically significant when ≤ 0.05.

3. Results

A total of 37 patients met the inclusion criteria for the study and had their charts reviewed. Of these, 16 patients (43.2%) had EGFR expression. The characteristics of patients grouped as positive and negative EGFR expression are shown in Table 1. Men represented the majority of patients in both groups. Tumor localization did not differ between groups, with approximately two-thirds located at the GEJ (Siewert I and II), and the remaining in the esophagus. Although well-differentiated tumors were less frequent in EGFR positive patients (44%) as opposed to 76% in EGFR negative, the difference was not statistically significant. Significant differences were found in pTNM staging. EFGR positive tumors presented higher scores either for pT ($T3 + T4$ = 94% versus 51%), pN involvement (94% versus 53%), or pM (57% versus 0%), in comparison with EGFR negative lesions. Accordingly tumor staging also differed between groups: all patients with positive EGFR belonged to stages III or IV, whereas most patients (62%) negative for EGFR had stage I or II lesions. EGFR expression was more frequent as higher



(a)

(b)

(c)

Figure 1: EGFR expression at immunohistochemistry (400X). In (a), a case of adenocarcinoma with negative EGFR. In (b) and (c), 2 different cases of adenocarcinoma with positive EGFR (brown staining).

was the pTNM staging (I and II = 0% versus III = 47% versus IV = 100%; $P < 0.001$).

Out of 37 patients, 4 died soon after the surgery due to operatory complications, including pneumonia and anastomotic leak. As presented in Figure 2, survival was significantly higher in EGFR negative patients compared to those who expressed EGFR (21.7 versus 10.5 months; $P = 0.001$).

4. Discussion

Adenocarcinoma of the esophagus and gastroesophageal junction is currently considered a public health problem, given its increasing incidence and poor survival [24]. Efforts to ameliorate outcomes, including optimization of prognostic markers, can be crucial to the management of patients with this condition. Prior studies have suggested that EGFR expression might be useful in predicting outcomes in patients with EA treated with different surgical techniques [18, 19]. The purpose of the present study was to confirm the utility of EGFR expression in the prognosis of patients with this malignant condition treated with a standardized surgical approach characterized by transhiatal esophagectomy.

The main findings of our study were (1) EGFR expression was related with more advanced lesions, with higher scores for both pTNM classification and tumor staging; (2) There was a trend to the degree of tumor differentiation be poorer in cases with EGFR expression; (3) survival was significantly shorter in the group of patients who expressed EGFR. Secondary findings included a relation between EGFR positivity and older age and predominance of GEJ compromising in spite of esophageal lesions.

In the current study, EGFR expression was found in nearly half of adenocarcinomas. This is in agreement with other studies, in which EGFR expression ranges between 32% and 64% [18, 19, 25, 26]. Besides its relatively high prevalence, EGFR expression was related with more advanced lesions, with higher scores either for tumor staging, nodal involvement, or metastasis. Furthermore, lesions with expressed EGFR showed poorer tumor differentiation. These findings have been demonstrated in other studies [18, 19], indicating that EGFR expression is a marker of more advanced tumors and therefore poorer prognosis.

Table 1: Characteristics of patients with and without EGFR expression.

	EGFR + ($n = 16$)	EGFR – ($n = 21$)	P
Age, mean ± SD	70.4 ± 9.0	61.2 ± 7.8	0.002
Men, n (%)	13 (81)	18 (86)	0.716
Tumor localization			
Esophageal, n (%)	5 (31)	6 (29)	0.999
Siewert I and II	11 (69)	15 (71)	
Tumor differentiation			
Well or moderate	7 (44)	16 (76)	0.086
Poor	9 (56)	5 (24)	
pTNM			
pT1	1 (6)	4 (19)	0.036
2	0	6 (29)	
3	11 (69)	9 (43)	
4	4 (25)	2 (9)	
pN negative	1 (6)	10 (47)	0.010
positive	15 (94)	11 (53)	
pM0	7 (43)	21 (100)	<0.0001
1	9 (57)	0	
Tumor staging, n (%)			<0.0001
I	0	3 (14)	
II	0	10 (48)	
III	7 (44)	8 (38)	
IV	9 (56)	0	

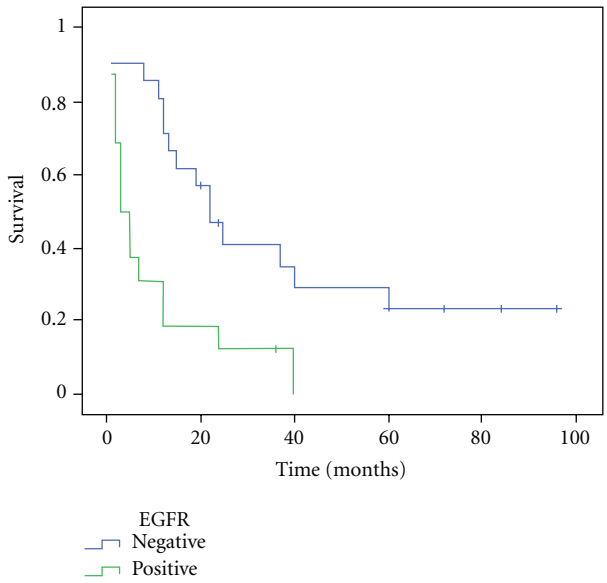


Figure 2: Survival curve (Kaplan-Meier) in patients with and without EGFR expression (4 patients excluded due to surgery-related mortality) ($P = 0.001$).

Survival was significantly shorter in the group of patients who expressed EGFR. This can be explained by several factors, including higher pTNM scores, poorer tumor differentiation and also older age in the group of patients with positive EGFR. These patients showed a trend in receiving more adjuvant treatment with radiochemotherapy after esophagectomy. This likely reflects advanced lesions, which usually require an aggressive approach in spite of surgical treatment [27, 28]. Prior studies have also suggested that EGFR expression is related with shorter survival [18, 19, 25, 26, 29]. It has been proposed that EGFR may participate in the carcinogenesis process of EA [30], based on the fact that EGFR may stimulate proliferation and migration of tumor cells [31, 32]. Further studies are needed to clarify this topic and assess a possible therapeutical benefit of anti-EGFR antibodies [33].

Contrasting with other studies, our patients were treated exclusively with transhiatal esophagectomy before providing tumor specimens for EGFR analysis. Thus, tissue evaluation did not suffer potential influences of other therapeutic modalities, including radiochemotherapy. In addition, EGFR analysis was carried out using immunohistochemistry, which has been considered a feasible technique for this purpose [34].

In conclusion, the current study assessed whether EGFR expression predicts tumor staging and survival in EA patients treated with transhiatal esophagectomy. We found that EGFR expression was related with older age, poor tumor differentiation, higher pTNM staging, and shorter survival in comparison with EGFR negative cases. These findings confirm EGFR expression as a prognostic marker in patients with adenocarcinoma of the esophagus and GEJ treated with a standardized surgical approach. Further studies are needed to test the hypothesis that endoscopic assessment of EGFR expression can be useful in the management of EA patients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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