Diploma Thesis

Gastric cancer and concomitant renal cancer: A systematic clinicopathological, immunohistochemical and molecular analysis

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and

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Graz, November 2011 Johannes Betge

Affidavit

I hereby declare that the following thesis has been written only by myself and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

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Graz, am …. *Unterschrift*

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Abstract

Objective: The frequency of gastric cancer in patients with renal cell carcinoma (RCC) is remarkably high in our area suggesting a common molecular basis. Our study aimed to characterize tumors and to analyze possible underlying molecular features in twelve patients with gastric cancer and concomitant RCC.

Methods: A histological and immunohistochemical analysis was performed, which included p53 protein expression, proliferative activity (MIB-1), mismatch repair status (hMLH1, hMSH2, hMSH6, PMS2) and E-cadherin expression for gastric cancers, which were additionally analyzed for Epstein-Barr-Encoded-RNA (EBER) by in-situ hybridization. Microsatellite instability was analyzed with a PCR multiplex system and capillary electrophoresis. *KRAS* mutations in codons 12 and 13 were tested by pyrosequencing.

Results: All patients had clear cell RCCs, ten of which were well differentiated and diagnosed in an early stage, while the patients' gastric cancers were generally poorly or undifferentiated and diagnosed in an advanced stage. Gastric cancers showed reduced E-cadherin staining in ten out of twelve cases. Two gastric cancers demonstrated loss of hMLH1 and PMS2, which was confirmed by molecular analysis showing a high degree of microsatellite instability. All RCCs were microsatellite stable. *KRAS* mutation was detected in one of the two instable gastric cancers, while none of the RCCs had *KRAS* mutations. Another gastric cancer was positive for EBV.

Conclusions: In conclusion, a coherent cause for gastric cancer and concomitant RCC, such as Lynch syndrome, a prominent role of *KRAS* mutation or EBV infection, was not found in our series. Other factors leading to a higher susceptibility for cancer will have to be explored to explain why people with RCC have a higher risk to develop gastric cancer in our area.

Zusammenfassung

Hintergrund: Das Auftreten von Magenkarzinomen bei Patienten mit Nierenzellkarzinomen ist an unserem Institut auffällig häufig, was auf einen ätiologischen Zusammenhang hindeuten könnte. Ziel der Studie war die Charakterisierung von Tumoren von zwölf Patienten mit Magen- und konkomitantem Nierenzellkarzinom und die Analyse möglicher zugrundeliegender molekularer Faktoren.

Methoden: Es wurde bei den Magen und Nierenzellkarzinomen eine histologische und immunhistochemische Untersuchung durchgeführt, welche die Expression von p53 sowie der mismatch-repair Proteine (hMLH1, HMSH2, hMSH6, PMS2), die proliferative Aktivität (MIB-1), und bei Magenkarzinomen zusätzlich die Expression von E-cadherin umfasste. Die Magenkarzinome wurden außerdem mittels *in situ*-Hybridisierung auf Epstein-Barr-Encoded RNA (EBER) untersucht. Mit einem PCR multiplex System und Kapillarelektrophorese wurden die Tumoren auf Mikrosatelliteninstabilität untersucht. *KRAS* Mutationen in Codons 12 und 13 wurden mittels Pyrosequenzierung analysiert.

Ergebnisse: Alle Patienten hatten ein klarzelliges Nierenzellkarzinom, davon waren zehn hochdifferenziert und wurden in einem frühen Tumorstadium diagnostiziert, während die Magenkarzinome der Patienten generell niedrig- oder undifferenziert und in fortgeschritteneren Stadien waren. Die Magenkarzinome zeigten einen Verlust der E-cadherin Expression in zehn von zwölf Fällen. Zwei Magenkarzinome zeigten einen Verlust von hMLH1 und PMS2, was durch eine hochgradige Mikrosatelliteninstabilität in der molekularen Analyse bestätigt wurde. Alle Nierenzellkarzinome waren mikrosatellitenstabil. *KRAS* Mutationen wurden in einem der zwei mikrosatelliteninstabilen Magenkarzinome gefunden, während keins der Nierenzellkarzinome *KRAS* Mutationen zeigte. Das Magenkarzinom eines Patienten war positiv für EBER.

Schlussfolgerungen: Eine zusammenhängende Ursache für die Entwicklung von Magenkarzinomen und konkomitanten Nierenzellkarzinomen, wie das Lynch Syndrom, eine bedeutende Rolle von *KRAS* Mutationen oder dem Epstein-Barr Virus konnte in unseren Patienten nicht nachgewiesen werden. Andere Faktoren, die zu einer erhöhten Empfindlichkeit für Krebserkrankungen führen, können dazu beitragen, dass Nierenzellkarzinompatienten in unserer Gegend ein höheres Risiko für die Entwicklung eines Magenkarzinoms haben.

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Glossary and Abbreviations

AD	Autosomal dominant
AEC	Aminoethylcarbazole; used as chromogen in immunohistochemistry; substrate of HRP
AJCC	American Joint Committee on Cancer
AP	Alkaline phosphatase
AWD	Alive with disease
BAT-25	Microsatellite marker
BAT-26	Microsatellite marker
BC	Breast cancer
BCIP	5-Bromo-4-chloro-3-indolyl phosphate, used as chromogen in immunohistochemistry; substrate of AP
BHD	Also referred to as *FLCN*; gene encoding folliculin, tumor suppressor; germline mutations are associated with Birt-Hogg-Dubé syndrome
BRAF	Proto-oncogene; coding for a serine/threonine-specific protein kinase; involved in MAPK/ERK pathway
BRCA1/2	Breast cancer 1 and 2; coding for proteins involved in DNA repair; certain mutations increase risk for breast- and other cancers
CCD	Charge coupled device
CDH1	Gene encoding E-cadherin; germline mutations are associated with hereditary diffuse gastric cancer
CNS	Central nervous system
CRC	Colorectal cancer
DAB	Diaminobenzidine; used as chromogen in immunohistochemistry; substrate of HRP
dNTP	Deoxyribonucleotide triphosphate
DOC	Died of other causes
DOD	Died of disease
EBER	Epstein-Barr encoded RNA
EBV	Epstein-Barr virus

F	Female
FAP	Familial adenomatous poliposis
FDGC	Familial diffuse gastric cancer
FFPE	Formalin fixed paraffin embedded
FGC	Familial gastric cancer
FH	Fumarate hydratase; heterozygous germline mutations are associated with hereditary leiomyomatosis and renal cell cancer syndrome
FIGC	Familial intestinal gastric cancer
G	Grade
Gly12Ala	Amino acid substitution of Alanine for Gycine due to mutation of codon 12 (in the *KRAS* gene)
Gly12Asp	Amino acid substitution of Aspartate for Gycine due to mutation of codon 12 (in the *KRAS* gene)
Gly12Val	Amino acid substitution of Valine for Gycine due to mutation of codon 12 (in the *KRAS* gene)
H&E	Heamatoxylin and eosin stain
HDGC	Hereditary diffuse gastric cancer
HNPCC	Hereditary nonpolyposis colorectal cancer; Lynch syndrome
hMLH1	Human MutL homolog 1; mismatch repair protein
hMSH2	Human MutS homolog 2; mismatch repair protein
hMSH6	Human MutS homolog 6; mismatch repair protein
HRP	Horseradish peroxidase
HRPT2	Also referred to as *CDC73*, mutations are associated with hyperparathyroidism-jaw tumor syndrome
Inh.	Inheritance
ISH	*In situ* hybridization
KRAS	Proto-oncogene; coding for V-Ki-ras2 / Kirsten rat sarcoma viral oncogene homolog; GTPase involved in signal transduction
LASB	Labeled streptavidin biotin method; biotin – streptavidin affinity is used to link streptavidin bound chromogenic enzymes and biotinylated antibodies in immunohistochemistry
M	Male

MIB-1	Monoclonal antibody to detect ki-67 antigen; cellular marker for proliferation
MONO-27	Microsatellite marker
MSI	Microsatellite instability
MSI-H	Microsatellite instability-high
MSS	Microsatellite stable
MW	Microwave
NED	No evidence of disease
NR-21	Microsatellite marker
NR-24	Microsatellite marker
NTP	Nitro blue tetrazolium chloride, used as chromogen in immunohistochemistry, substrate of AP
OG	Oncogene
PBS	Phosphate buffered saline
PMS2	Postmeiotic segregation increased 2 – mismatch repair protein
POP 6	Performance optimized polymer 6
PPi	Pyrophosphate
RCC	Renal cell carcinoma
TP53	Gene encoding p53; regulator of cell cycle, tumor suppressor
TS	Tumor suppressor
TSC1/2	Genes encoding tuberous sclerosis protein 1 (hamartin) and 2 (tuberin); complex acts as tumor suppressor; germline mutations lead to tuberous sclerosis
UICC	Union internationale contre le cancer
VHL	Tumor suppressor gene; germline mutations are associated with von Hippel-Lindau syndrome
WB	Water bath
WHO	World Health Organization
Yr, yrs	Year, years

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1 Introduction

Patients with multiple malignancies represent an attractive research topic. The analysis of different tumors occurring in a single patient may lead to detection of hereditary causes for cancer or cancer syndromes and may improve understanding of general molecular pathologic principles of carcinogenesis.

1.1 Renal cell carcinoma: definition, epidemiology, risk factors, classification

1.1.1 Epidemiology and definition

About 2% of all human cancers arise within the kidney, amounting to approximately 190,000 newly diagnosed cases worldwide each year. Incidences vary geographically, the highest rates are found in central Europe and among the black population of North America (1,2). Worldwide, incidences of renal cancer have increased during the last 30 years. Most cases present in the fifth to seventh decade of life (3,4).

90% of kidney cancers in adults are renal cell carcinomas, which arise from the epithelium of the renal tubules (2). The second most common type of kidney cancer is urothelial cell carcinoma of the pelvicaliceal system (4). Other neoplasms located in the kidneys include nephroblastic tumors, mesenchymal tumors, mixed mesenchymal and epithelial tumors, neuroendocrine tumors, hematopoietic and lymphoid tumors, germ cell tumors and metastases (2).

1.1.2 Risk factors

Major risk factors for renal cell carcinoma include tobacco smoking, obesity and hypertension. End stage renal disease and diabetes mellitus have also been associated with a higher risk for renal cancer (1,2). Exposure to occupational and environmental factors like asbestos, arsenic compounds, polycyclic aromatic hydrocarbons and others have been suggested to increase the risk for kidney can-

cer. However, consistent evidence has not been established (1,2,4). Moreover, family history is a risk factor for renal cancer (1,2). Additionally, some hereditary cancer syndromes associated with renal cancer, like von Hippel-Lindau syndrome, Birt-Hogg Dubé syndrome, hereditary papillary renal cancer and others have been identified, which account for about 2-3% of renal cell carcinomas (4).Those will be discussed below.

1.1.3 Classification

Renal cell carcinomas are usually assessed according to the WHO classification: Clear cell carcinoma represents the most common histological subtype. The tumor is believed to originate from cells of the proximal tubule. It is characterized by large uniform cells with clear or eosinophilic cytoplasm, embedded within a delicate network of small blood vessels. The cells are rich in lipid and glycogen, which is dissolved during histologic work-up causing the “clear” aspect of the cytoplasm, while macroscopically the tumors show a golden-yellow cut surface. Cysts are common in clear cell carcinomas, but if the tumor is entirely composed of cysts with septa containing clear cells, it is referred to as mulitilocular cystic renal cell carcinoma (2,5-7).

The second most common renal cell cancer is the papillary carcinoma, which is characterized by papillary or tubulopapillary architecture. Two subtypes have been described: Type 1 typically consists of papillae covered with a single layer of small tumor cells. It metastasizes later in the disease course and has a comparably good prognosis. This type tends to be multifocal, particularly in end stage kidney diasease. Type 2 tumors have large cells with eosinophilic cytoplasm and pseudostratified nuclei. They often present with higher nuclear grade and have a poor prognosis (2,5,6).

Chromophobe renal cell carcinomas show large pale cells with prominent cell membranes. They have a relatively good prognosis with mortality less than 10% (2,5,6,8). Collecting duct tumors are derived from the principal cells of the Bellini duct. They are typically located in the central region of the kidney, often show a tubular or tubulopapillary growth pattern with irregular glands infiltrating

renal parenchyma and have an extremely poor prognosis. Medullary carcinoma is a very rare tumor with poor prognosis that is associated with sickle cell trait. Other rare variants of renal cell carcinoma are renal carcinomas associated with Xp11.2 translocations / *TFE3* gene fusions, renal cell carcinoma associated with neuroblastoma, and mucinous tubular and spindle cell carcinoma (2,5,6).

1.2 Gastric cancer: definition, risk factors, epidemiology, classification

1.2.1 Definition and risk factors

Gastric adenocarcinoma is a malignant epithelial tumor of the stomach showing glandular differentiation (9).

Helicobacter pylori infection is classically associated with gastric carcinoma via the precursor lesions multifocal atrophic gastritis, intestinal metaplasia and dysplasia. Bile reflux after Billroth II operation represents another well established risk factor, as well as dietary and life style factors like tobacco smoking, exposure to nitrosamines, high salt intake and smoked or cured meats or fish, while fresh fruits and vegetables may lower the risk. These factors are especially associated with an increased risk for intestinal type cancer. Moreover, familial risk plays a role, especially for diffuse type gastric cancer (compare below) (9-11).

1.2.2 Epidemiology

Gastric cancer is the fourth most common cancer worldwide. In the year 2008 it accounted for estimated 8% of new cancer cases and 10% of cancer deaths worldwide (1,12). In general, the incidence of gastric cancer shows wide geographic differences. Moreover, the rate has decreased in the last decades, which might be related to changes in diet, i.e. refrigerated, fresh foods instead of salted and preserved foods, and reduced incidence of chronic H. pylori infection in many parts of the world (1,12).

1.2.3 Classification

Gastric carcinomas are most commonly classified according to Laurén (13) and the WHO (9).

The Laurén classification differentiates gastric cancer into two types reflecting morphology (intestinal vs. diffuse), but the classification also has clinicopathological, pathogenetic and prognostic relevance. On gross inspection, the intestinal type of gastric carcinoma is well delineated. The tumor is characterized by clear-cut glandular differentiation. It is often accompanied by intestinal metaplasia and typically follows atrophic gastritis, which are therefore regarded as precursor lesions. In contrast, the diffuse type of gastric cancer consists of diffusely infiltrating, poorly cohesive cells with little or no gland formation. Loss of E-cadherin expression by tumor cells is a characteristic feature. In general, diffuse type gastric cancer is not associated with atrophic gastritis or intestinal metaplasia and it may occur more commonly in young patients and in areas with low prevalence (9,11,13,14). Different genetic pathways have been hypothesized for intestinal and diffuse gastric cancer, however most of the observed genetic alterations have been found in both types (15).

According to the WHO classification, tumors are classified by their predominant histological pattern: Tubular adenocarcinomas contain ‘dilated or slit-like and branching tubules varying in their diameter’ (9). Papillary adenocarcinomas are exophytic tumors that have digitiform branches consisting of lined cuboidal or cylindrical cells and fibrovascular tissue. Mucinous adenocarcinomas have extracellular mucin pools in more than 50% of the tumor, tumor cells from glands, chains or irregular cell clusters. Signet ring cell carcinomas consist of more than 50% isolated cells or small groups of cells containing intracytoplasmatic mucin. Rare variants of gastric adenocarcinoma are adenosquamous carcinomas, which combine adenocarcinoma and squamous cell carcinoma features, pure squamous cell carcinomas and undifferentiated carcinomas, which lack any differentiated features except epithelial differentiation (9).

1.3 Familial and hereditary gastric cancer

Familial clustering is observed in approximately 10% of gastric cancer cases, but only 1-3% of these are hereditary (16). Cases with familial clustering are classified into familial diffuse gastric cancer (FDGC), including hereditary diffuse gastric cancer (HDGC), a cancer syndrome caused by germline mutation in the E-cadherin (*CDH1*) gene (16-18), as well as familial intestinal gastric cancer (FIGC) or, if the histology is unknown, familial gastric cancer (FGC)(19). HDGC predisposes to poorly differentiated, diffuse gastric cancer presenting typically before the age of 50. For women, the risk of lobular breast cancer is also increased (14,16,17).

Moreover, gastric cancer may be observed as part of a hereditary tumor syndrome with main localization other than the stomach. Thus, germline mutations of the *BRCA1* and *BRCA2* genes do not only increase the risk for breast and ovarian cancer, but do also increase the incidence of stomach, pancreas, prostate, and colorectal cancer (20). Especially *BRCA2* has been associated with a higher risk for gastric cancer (21).

Li-Fraumeni syndrome is related to mutations of the tumor suppressor gene *TP53*. Affected patients may suffer from a wide spectrum of tumors, most frequently early onset breast cancer, soft tissue or bone sarcoma, adrenocortical cancer and brain tumors, but also hematologic malignancies, melanoma, lung, ovary, colon and gastric cancer (9,22-24).

Gastric cancer may also occur in the setting of a gastrointestinal polyposis syndrome. Patients with familial adenomatous polyposis (FAP) develop multiple colorectal polyps and almost certainly colorectal cancer before the age of 60. Duodenum, pancreas and the hepatobiliary system are the most common extracolonic sites of malignancy in FAP, but the syndrome is also related to thyroid cancer and gastric cancer (incidence 0.5%) (21,25). Individuals affected by Peutz-Jeghers syndrome harbor multiple hamartomatous polyps in their gastrointestinal tract, show mucocutaneous pigmentation and have a high risk for colorectal, gastric, breast, pancreatic and small intestinal neoplasms (26). In juvenile polyposis syndrome, increased risks for gastric and other gastrointestinal cancers have similarly been observed (21).

Finally, gastric cancer represents a well known extra-colonic manifestation of the Lynch (HNPCC) syndrome (Table 1) (21,27-29). Neoplasms occurring as part of the Lynch syndrome are characterized by microsatellite instability (MSI) caused by germline mutations in mismatch repair genes (28,30).

Table 1: Lynch syndrome-related tumors according to the revised Bethesda criteria (28)

- Colorectal carcinoma
- Endometrial carcinoma
- Gastric carcinoma
- Ovarian carcinoma
- Pancreatic carcinoma
- Carcinoma of the upper urinary tract (ureter and renal pelvis)
- Carcinoma of the billiary tract
- Carcinoma of the small bowel
- Brain tumors
- Sebaceous gland adenomas
- Keratoacanthomas

1.4 Gastric cancer and renal cell carcinoma

None of the syndromes mentioned above have been described to increase the risk for renal cell carcinoma. Nevertheless, the association of primary gastric and primary renal cancer has been presented in several case reports (31-44). Likewise, in the renal cell carcinoma database of our institute, we found 12 patients, who, in addition to renal cell carcinoma, also experienced gastric cancer, either syn- or

metachronously. The observed incidence was nearly twice as high as documented in the national cancer registry regarding the gastric cancer incidence in Austria (45).

1.5 Aim

Therefore, the present study aimed to evaluate patients with gastric and concomitant renal cancer. It is the first study to systematically assess this association.

First of all, we characterized the clinical and pathological presentation of these patients, analyzed epidemiologic and clinical data and presented histopathological and immunohistochemical features.

In a second step, in order to investigate possible underlying genetic as well as infectious factors in the pathogenesis of both cancer types, a thorough molecular analysis with respect to MSI, *KRAS* mutation and Epstein-Barr-Encoded-RNA (EBER) status was performed.

2 Materials and Methods

2.1 Patient selection

The renal cell carcinoma database of the Institute of Pathology, Medical University of Graz, Austria, covers 2082 patients who underwent radical or partial nephrectomy between January 1984 and September 2005. Included are 1180 males and 902 females (ratio 1.3:1, Figure 1). Patients in the renal cell carcinoma database had a mean age of 62 years (median 63, range 9-88), age distribution is shown in Figure 2. Distribution of histological subtypes is shown in Figure 3.

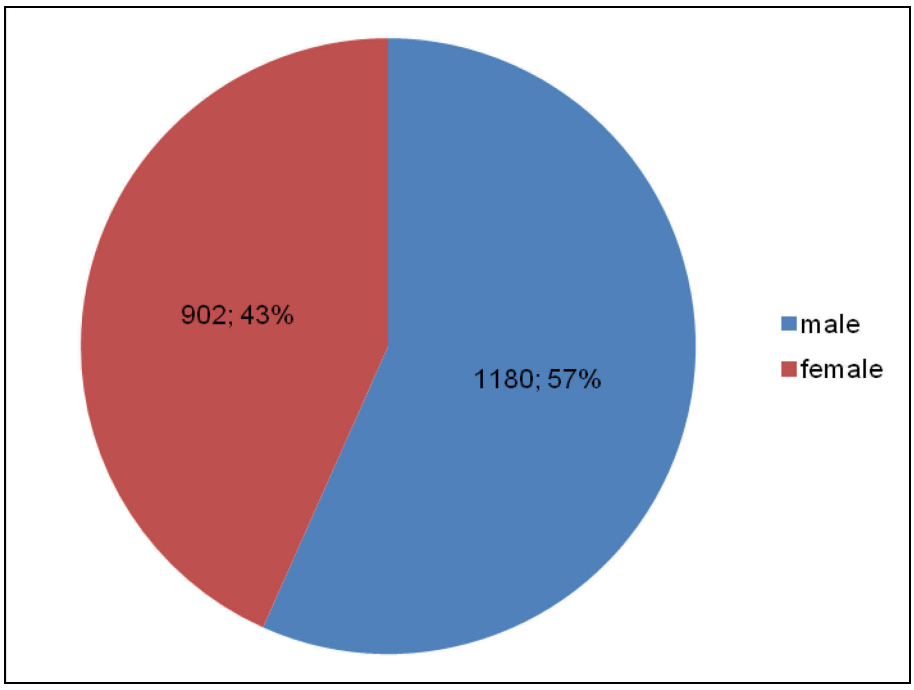

Figure 1: Gender of patients in the renal cell carcinoma database

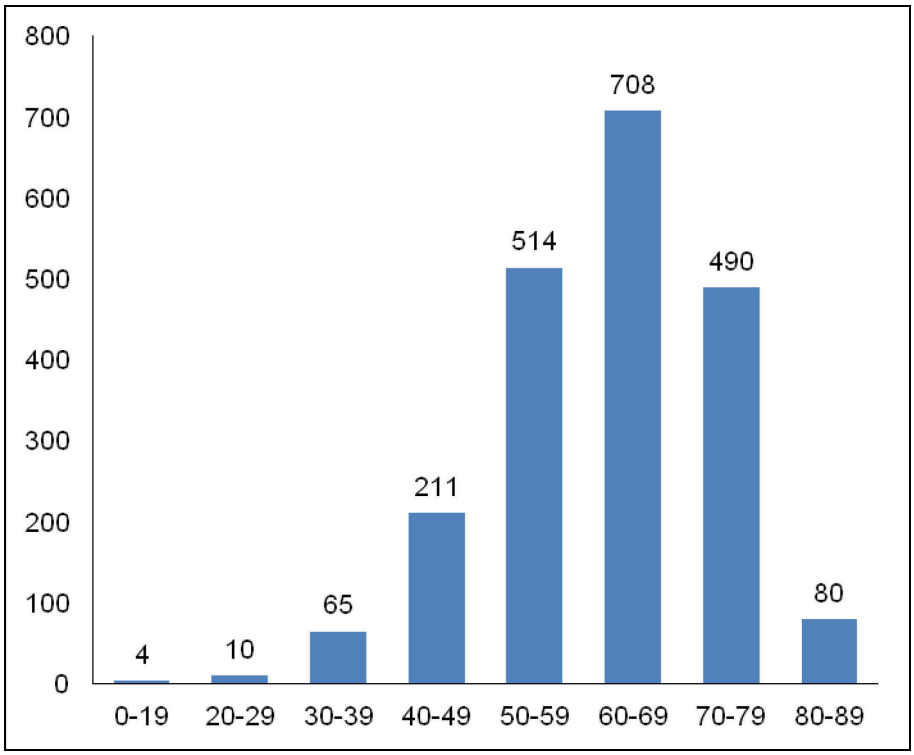

Figure 2: Age distribution of patients in the renal cell carcinoma database

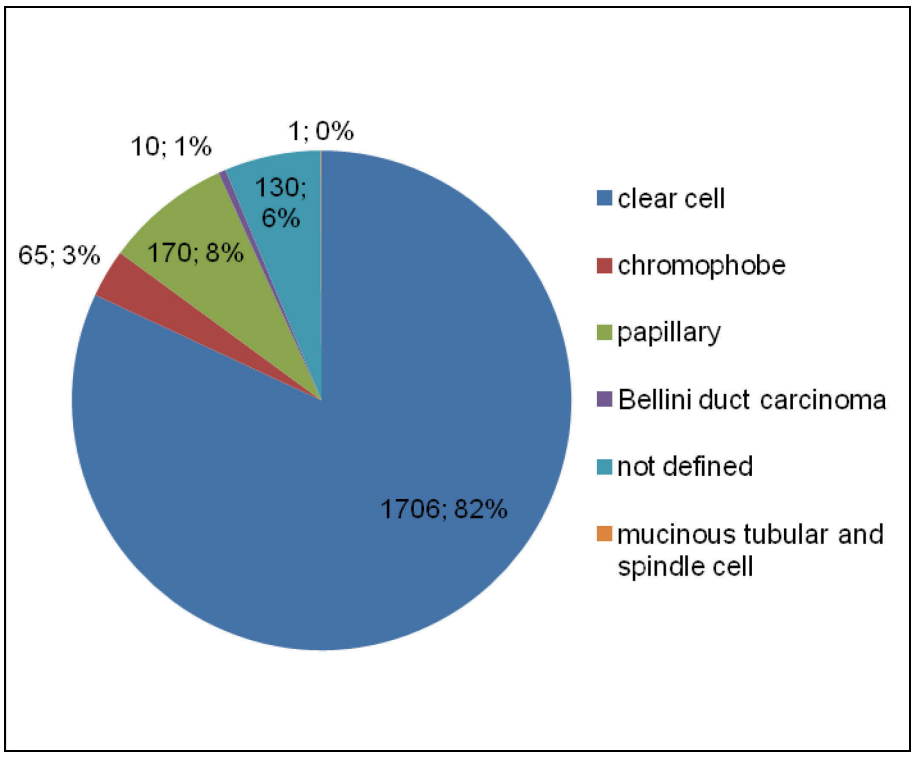

Figure 3: Histological types of renal cell carcinoma in the renal cell carcinoma database

The gastric cancer database of the Institute of Pathology, Medical University of Graz, Austria, covers 3072 patients who underwent total or partial gastrectomy between January 1984 and December 2008. Included are 1689 males and 1378 females (ratio 1.2:1, Figure 4). Patients in the gastric cancer database had a mean age of 68 years (median 69, range 11-97), age distribution is shown in Figure 5.

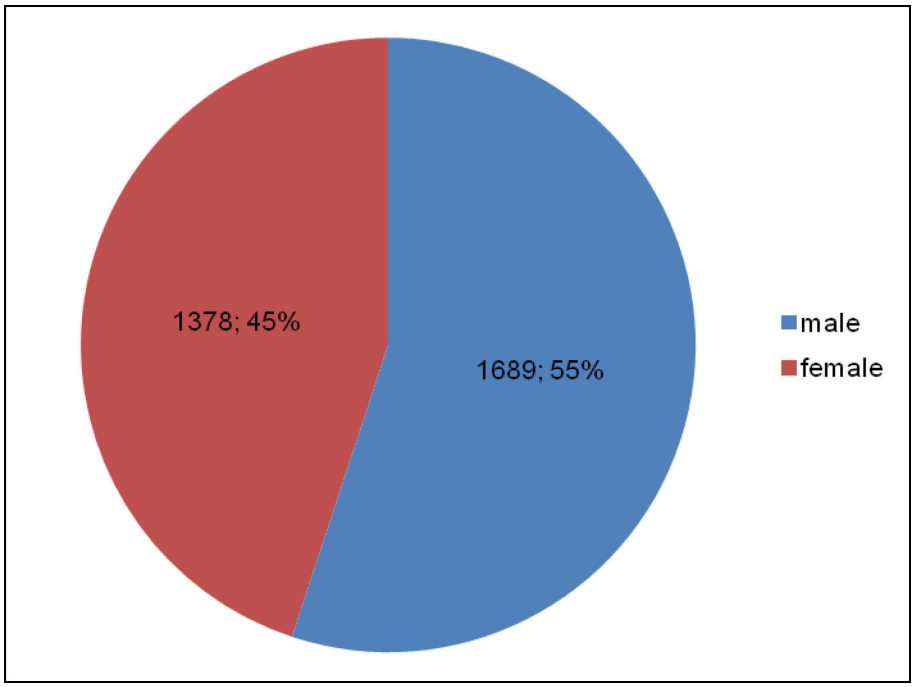

Figure 4: Gender of patients in the gastric cancer database

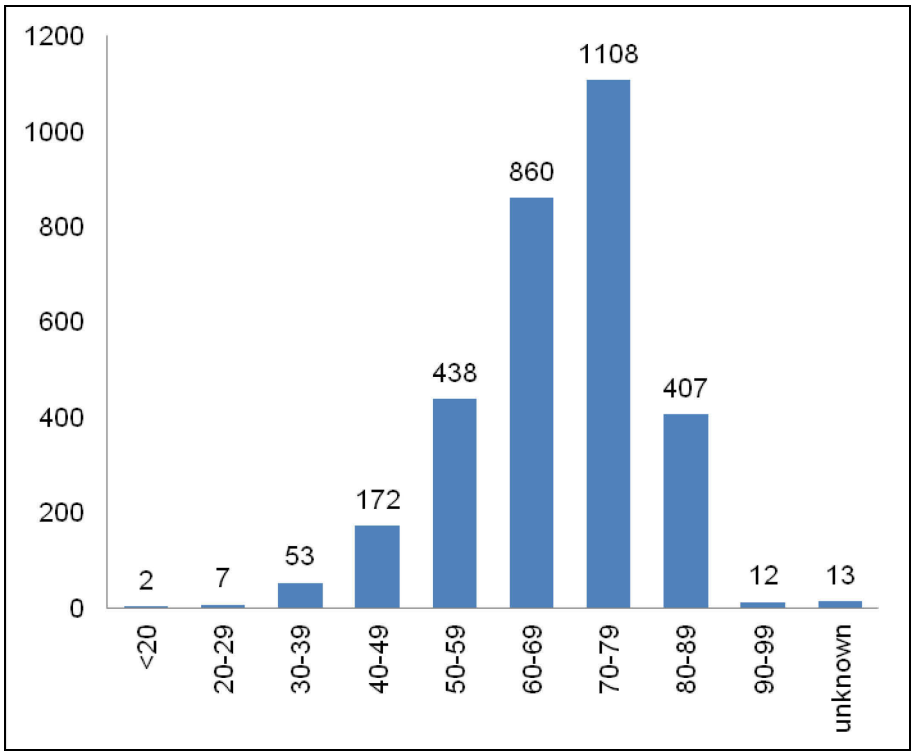

Figure 5: Age distribution of patients in the gastric cancer database

A systematic search of the renal cell carcinoma database was performed to identify those with syn- and/or metachronous diagnosis of cancer in gastric biopsies or resection specimens. The medical records of these patients were reviewed in order to differentiate between patients with primary gastric cancer and those with secondary gastric involvement by renal cell carcinoma.

Patients with renal cell carcinoma metastatic to the stomach were presented in a previous publication (46), in which the clinical significance of this rare finding was described. During analysis, another twelve patients with syn- and/or metachronous occurrence of both primary gastric and primary renal cell carcinoma were identified who represent the scope of the current analysis.

Clinicopathological and follow-up data of the twelve patients were analyzed in detail by chart review and interviewing attending physicians if possible. Basic personal data, such as patient age and gender were compared with that of patients suffering from either gastric or renal cancer contained in the computerized renal cell carcinoma and/or gastric cancer databases of our institution, respectively.

2.2 Ethics board approval

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Institutional Review Board approval was obtained from the Ethic's Committee of the Medial University of Graz, Austria.

2.3 Histopathology

Tissues were routinely fixed in 4% buffered formalin, embedded in paraffin and stained with H&E. All tumor probes were carefully reassessed, paying special attention to tumor stage and grade as well as histological subtype of both renal and gastric cancers. Tumors were staged according to the 7th edition of the American Joint Committee on Cancer – Union Internationale Contre le Cancer (AJCC – UICC) Tumor-Node-Metastasis (TNM) classification (47). Tumor grades were assessed following the WHO guidelines on renal (2) and gastric (9) cancers, respectively. Gastric cancers were additionally classified according to the Laurén classification (13).

2.4 Immunohistochemistry

2.4.1 Principle

Immunohistochemistry is used to detect the expression and localization of target antigens in tissue samples with the help of specific antibodies. In this study, detection systems using secondary antibodies against the primary antibody (indirect method) and horseradish peroxidase (HRP) as chromogenic enzyme were applied. The secondary antibody was linked to the HRP ether with a polymer-backbone covering multiple HRP enzymes, or secondary antibodies were labeled with biotin to allow binding of streptavidin-enzyme complexes (labeled streptavidin-biotin method, LASB). Both methods lead to amplification of the signal. Diaminobenzidin (DAB) and Aminoethylcarbazole (AEC) were used as

chromogens. (compare below and Table 2). Before immunostaining, epitope retrieval is necessary to unmask the antibody targets, since formaldehyde causes cross-linkage between proteins or between proteins and nucleic acids, thereby changing the three-dimensional structure of proteins (48).

2.4.2 Procedure

Slides were processed with automated slide preparation systems. Commercially available antibodies were used and diluted as specified in Table 2.

Tissues had been routinely fixed in 4% buffered formalin and embedded in paraffin. Four micrometer sections were cut with a microtome and mounted onto glass slides.

Slides stained with antibodies against p53, E-cadherin, hMLH1 and PMS2 were processed using the Dako TechMate™ 500 automated staining system (Dako, Glostrup, Denmark). For these antibodies, deparaffinization was done manually, using a xylene bath for 10 minutes and rehydration in a graded alcohol series including immerging in xylene 50% / ethanol 50%, absolute ethanol, 90%, 80%, 70% and 50% ethanol, respectively, followed by 30 seconds of rehydration in phosphate buffered saline (PBS). Epitope retrieval was performed and standardized for each antibody, using microwave or water bath treatment (Table 2). Slides were cooled down for 20 minutes to room temperature and washed with PBS before being placed in the autostainer instrument.

MIB-1, hMSH2 and hMSH6 staining was performed using the Benchmark XT automated staining system (Ventana Medical Systems, Tucson, USA), which includes automated deparaffinization as well as epitope retrieval using proteinase digestion and different buffer solutions (Table 2).

Tissues were stained according to protocols standardized for each antibody and commercially available detection systems were used for visualization (compare Table 2): The Dako REAL™ Detection System (Catalog No K5001, Dako) and the iView DAB detection kit (Catalog No 760-091, Ventana) both use biotinylated link antibodies directed against the primary mouse or rabbit antibodies and streptavidin conjugated HRP (LASB method), while the *ultra*View™ Universal

DAB Detection Kit (Catalog No 760-500, Ventana) and the the EnVision+ (HRP rab/mouse) detection system (Catalog No K5007, Dako) are polymer / multimer based detection systems.

Slides stained with DAB as a chromogen were dehydrated in 80% and 100% alcohol. Slides were counterstained with hematoxylin and then mounted with a coverslip using Entellan mounting medium (Merck, Darmstadt, Germany). Slides stained with AEC were washed with Aqua dest. and mounted with a coverslip using Aquatex mounting medium (Merck), since AEC is soluble in alcohol.

Table 2: Antibodies used for immunohistochemical staining

Antibody	Source	Clone	Dilution	Epitope Retrieval	Detection system	Chrmo-gen
p53	DAKO, Glostrup, Denmark	DO-7	1:100	WB, Buffer pH 6.0	B	AEC
MIB-1	Ventana, Tucson, AZ, USA	K-2	Ready to use	Ventana iView Kit	A	DAB
E-cadherin	Zymed, San Francisco, CA, USA	4A2C7	Ready to use	MW, Buffer pH 9.0	C	AEC
hMLH1	Biocare, Con-corde, CA, USA	G168-15	1:50	MW, Buffer pH 9.0	C	DAB
hMSH2	Ventana	G219-1129	1:50	Buffer CC1 stand-ard	D	DAB
hMSH6	Biocare	BC-44	1:50	Buffer CC1 mild	D	DAB
PMS2	BD Biosci-ences, San Jose, Ca, USA	A 16-4	1:50	MW, Buffer pH 6.0	C	DAB

A, Ventana iView DAB; B, Dako REAL Detection System K5001; C, Dako EnVision+ (HRP rab/mouse) K5007; D, Ventana *ultra*View DAB 760-500; DAB, Diaminobenzidine Dako (K5001); AEC, Aminoethylcarbazole Dako (S2367); Buffer pH9: Target Retrieval Solution Dako (S2367); Buffer pH6.0: Epitope Retrieval Solu-tion Dako (K5207); WB, Water Bath; MW, Microwave; CC1, Cell conditioning 1 Ventana (950-124 SL)

2.4.3 Evaluation

Immunoreactivity was independently assessed by two investigators and discrepancies were resolved by simultaneous re-examination of the slides using a double-headed microscope. Regarding p53 and MIB-1, positivity was identified as brown nuclear staining. The number of positive cells (labeling index) was determined by counting positive cells per 100 cancer cells, and immunoreactivity was semiquantitatively assessed as follows: negative (<10%), weak or 1+ (11-20%), moderate or 2+ (21-50%), and high or 3+ (>50%). The amount of E-cadherin staining was evaluated in relation to staining of non-neoplastic mucosa and was assessed as reduced, when up to 50% of cancer cells lost specific membraneous labeling, markedly reduced, when more than 50% lost specific membraneous labeling, negative or positive. Regarding the four MMR proteins (hMLH1, hMSH2, hMSH6, PMS2), staining was recorded as either present (positive) or absent (negative).

Slides of a colorectal cancer known to exhibit high p53 as well as E-cadherin expression served as positive controls for p53, E-cadherin and MIB-1 immunostaining, respectively. Intratumoral lymphocytes served as positive controls for MMR proteins. Negative controls included omission of the primary antibodies and incubation with Dako REAL^{TM} Antibody Diluent (No. S2022, Dako).

2.5 In situ hybridization

2.5.1 Principle

In situ hybridization uses small probes of nucleic acid to localize a specific complementary DNA or RNA segment on histological slides. The probes may be directly labeled with a reporter molecule or, as done here, visualized with the help of a primary antibody against the labeled probe and a secondary antibody linked to a chromogenic enzyme.

In situ hybridization was performed to detect EBV infection, using the INFORM EBER assay (Ventana). EBERs are RNAs that are expressed at a very

high level in EBV infected cells. RNA *in situ* hybridization is currently the gold standard for EBV diagnosis in tumor tissue (49). The INFORM EBER test includes a mixture of oligonucleotide probes complementary to EBERs.

2.5.2 Procedure

Four micrometer formalin fixed, paraffin embedded sections were processed with an automated slide stainer system (Benchmark XT, Ventana) and an *in situ* hybridization detection system (ISH iVIEW Blue Detection Kit, Ventana). Each 20µl of iVIEW Blue anti-fluorescin antibody (mouse), biotinylated Immunoglobulin (goat; anti-mouse), enhancer, streptavidin conjugated alkaline phosphatase, NBT, BCIP, protease 1 and 10 µl of iVIEW red counterstain II (all Ventana), as well as 20 µl (12U) of Epstein-Barr virus encoded RNA probe (INFORM EBER Probe, Ventana) were loaded into the reagent carousel and slides were placed in their position in the autostainer instrument. A standardized protocol was run: After automated deparaffinization and rehydration, tissues were treated with proteinase K and then hybridized with the fluorescein-labeled EBER oligonucleotide probe. The hybridized probe was visualized with the mouse antifluorescein IgG primary antibody binding to the fluorescein-labeled EBER probe and the biotinylated goat IgG secondary antibody formulation binding to the primary mouse antibody. Streptavidin conjugated alkaline phosphatase then bound the biotin and was used as a chromogenic enzyme, which generated a blue nuclear signal using BCIP/NBT as chromogen. Slides were then thoroughly washed with soap water and after an acetone bath, slides were mounted a coverslip using Entellan mounting medium.

2.5.3 Evaluation

Slides were evaluated using light microscopy. A nasopharyngeal carcinoma was used as a positive control.

2.6 DNA extraction

2.6.1 Principle

DNA from formalin fixed paraffin embedded (FFPE) tissue was extracted using the Qiagen QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). The procedure is based on proteinase digestion of the tissue followed by the DNA binding to a silica membrane and spin procedures for purification.

2.6.2 Procedure

Paraffin blocks were pre-cooled on a cooling plate. Of each paraffin block, two to eight 2-4µm thick sections were cut with a microtome and placed into a 1.5 ml tube. The material was deparaffinized with xylene and ethanol: 1000µL of xylene were added to the tube containing the paraffin material, the sample was mixed by pulse vortexing for 15 seconds, centrifuged at full speed for 8 minutes and then the supernatant was removed. These steps were repeated twice with xylene, and thereafter the same procedure was done twice using 1000µL ethanol instead of xylene.

The ethanol was evaporated in a heating block, before the pellet was re-suspended in 180µl Buffer ATL and 20µL Proteinase K (both Quiagen) and incubated over night at 56°C in a shaking heating block for lysis. The next morning, 200µl Buffer AL was added and the mix was incubated for 10 minutes at 70°C, then 200µl ethanol was added which formed a white precipitate.

The whole lysate was loaded into the QIAmp spin columns. Binding of DNA to the silica membrane and disposal of the other cell particles was accomplished by centrifugation at 8.000rpm for 1 minute, discharging of the filtrate, and two washing steps, first with Buffer AW1, then with Buffer AW2 (both Qiagen), each step including centrifugation and then discarding the filtrate.

The silica membrane was dried by centrifugation, and elution was done with nuclease free water by centrifugation at 8.000 rpm for one minute, using an elution volume of 50µl.

DNA concentration was measured using NanoDrop (Thermo Fisher Scientific, Wilmington, USA)

2.7 Microsatellite analysis

2.7.1 Principle

Microsatellites consist of repeating DNA segments of 2-6 base pairs. MSI is characterized by differences in the length of microsatellites due to insertions or deletions during DNA replication in the setting of mismatch repair deficiency. MSI was investigated using the Promega Microsatellite Analysis System version 1.2 (Promega, Mannheim, Germany), which is a PCR multiplex system using fluorescently labeled primers: 5 mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, MONO-27) to determine MSI, since mononucleotide repeats are prone to defects during replication, and 2 pentanucleotide repeat markers (Penta C and Penta D) for internal control (50).

2.7.2 Procedure

Two separate analyses using 50ng and 100ng of DNA per sample were performed, respectively. This was a considerably higher DNA concentration than recommended by the manufacturer due to the limitations of DNA quality from FFPE tissue. The used DNA concentrations were determined by a validation process of the method.

For each tested sample, 0.15µl AmpliTaq Gold DNA polymerase (5u/µl; Applied Biosystems), 1µl Gold Star 10x Buffer, 1µl of MSI 10x Primer Pair Mix, 5.85µl nuclease-free water (all Promega) and 2µl of template DNA were used, as recommended by the manufacturer. DNA from K562 cells served as positive amplification control, nuclease-free water as negative control.

Amplification was done using the GeneAmp PCR System 9700 (Applied Biosystems, Vienna, Austria) with the following cycling profile (Table 3):

Table 3: Amplification profile for MSI PCR

Temperature	Time	Cycles
95°C	11 minutes	
96°C	1 minute	
ramp 100% to 94°C	30 seconds	
ramp 29% to 58°C	30 seconds	10 cycles
ramp 23% to 70°C	1 minute	
ramp 100% to 90°C	30 seconds	
ramp 29% to 58°C	30 seconds	20 cycles
ramp 23% to 70°C	1 minute	
60°C	30 minutes	
4°C	soak	

PCR products were separated by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). 20µl of loading cocktail, including internal lane standard 600 for standardization of the analysis and Hi-Di formamide (Applied Biosystems) for stabilizing the (denaturated) DNA was used for each well with 1,5 µl of amplified sample. Denaturation was done by heating for 3 minutes at a temperature of 95°C and then chilling down to 4°C for 3 minutes. A 50 cm capillary array and performance optimized polymer 6 (POP 6, Applied Biosystems) were used for the runs to ensure optimal separation. Injection voltage was set 3kV and injection time 10 seconds.

2.7.3 Data analysis

The results were analyzed with GeneMapper software v3.2. According to the instructions, a shift in allele size of more than 3 base pairs or more was noted MSI positive. MSI at 2 mononucleotide loci was reported as MSI-high (MSI-H) and no instability at any of the loci tested as microsatellite stable (MSS).

2.8 KRAS mutation analysis

KRAS mutations in exon 1, codons 12 and 13 were tested by pyrosequencing using the Pyromark Q24 *KRAS* kit v2.0 (Qiagen).

2.8.1 Principle of pyrosequencing

The basic principle of pyrosequencing is detection of pyrophosphate (PPi) during DNA synthesis, which is released when a nucleotide is incorporated into the DNA chain by DNA polymerase (DNA_n + dNTP → DNA_{n+1} + PPi). In an enzymatic reaction cascade, PPi (in the presence of Adenosine-5-phosphosulfate) is converted to ATP by ATP-sulfurylase, and this ATP provides energy for the enzyme luciferase to oxydize luciferin, which also generates light (compare Figure 6). The four different deoxyribonucleotide triphosphates (dNTPs) are added to the reaction in a certain order (nucleotide dispensation order). If a dNTP is not incorporated, it is degraded by the enzyme apyrase, if it is incorporated, the light emission is detected by a charge coupled device (CCD) camera, so the sequence of the template DNA can be determined as the complementary DNA strand builds up (compare Figure 6) (51).

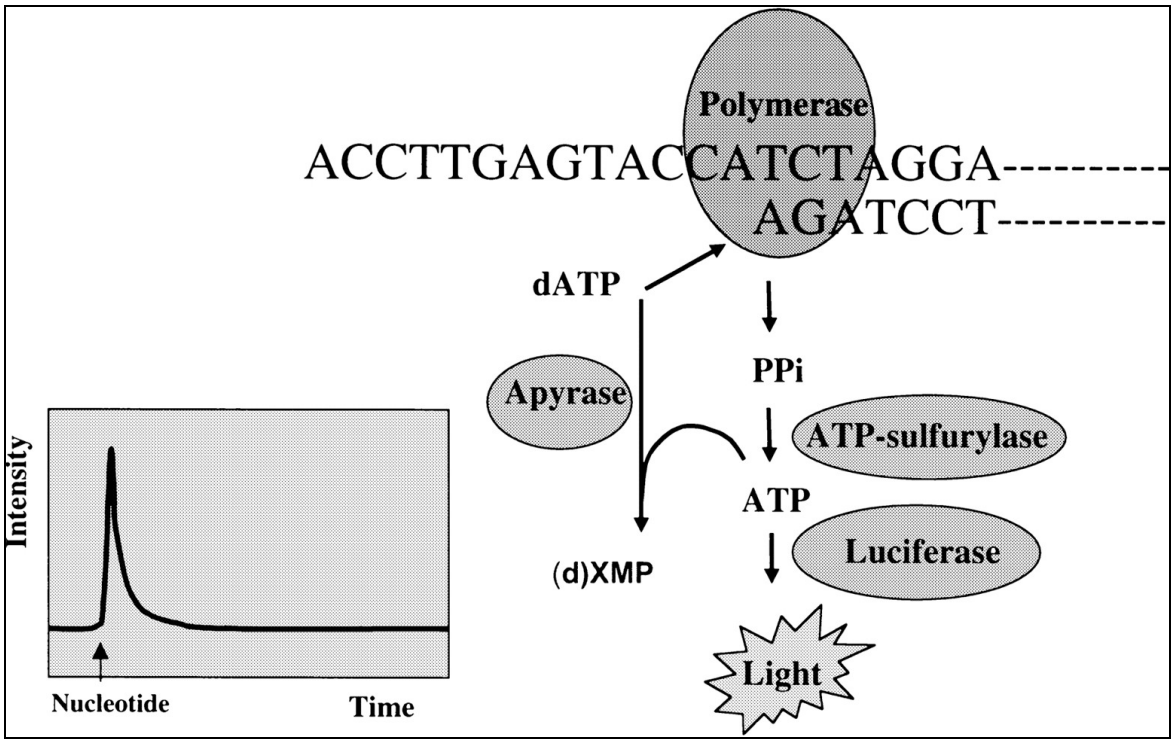

Figure 6: Schematic representation of the progress of the enzyme reaction in liquid-phase pyrosequencing.

Compare text for explanation. ©2001 by Cold Spring Harbor Laboratory Press. (51)

2.8.2 Procedure

PCR amplified, single stranded DNA spanning codons 12 and 13 is needed as template for pyrosequencing. Two analyses, using 20ng and 40ng of DNA respectively were performed.

The amplification was carried out in a 25µl reaction volume, containing 5µl of DNA solution (20ng or 40ng, respectively), 0,5µl of codon 12+13 biotinylated reverse primers, 0,5µl forward primer, 2,5µl Qiagen buffer, 0,2µl of each dNTP, and 0,16µl Qiagen Taq polymerase. The amplification profile was 15 min at 95°C; 45 cycles with 20 sec at 95°C, 30 sec at 53°C and 20 sec at 72°C; followed by 5 min at 72°C. Biotinylated primers are used to bind streptavidin for preparing single stranded DNA. 8µl of the PCR products were analyzed by electrophoresis in a 3% agarose gel to confirm successful amplification.

To prepare single stranded DNA, the PCR products were immobilized with streptavidin sepharose beads and purified with the PyroMark Q24 Vacuum Work-

station. 25µl pyrosequencing primer was annealed to the purified single-stranded PCR product. Pyrosequencing was conducted using the nucleotide dispensation order TACGACTCAGATCGTAG.

2.8.3 Data analysis

Results were analyzed with PyroMark Q24 software. GGT represents the wild type of codon 12, GGC the wild type of codon 13.

3 Results

3.1 Clinical data / outcome analysis

3.1.1 Epidemiologic data

The incidence of gastric cancer in renal cell carcinoma patients included in the renal cell carcinoma database of our institution was 27 / 100,000, compared with the gastric cancer incidence of 14 / 100,000 recorded in the Austrian cancer registry for the same study period (23).

Of the twelve patients, eight were male, four female. Gastric cancer was diagnosed at a mean age of 66 (median 65, range 41-78) years, compared with 68 years (median 69, range 11-97) in the gastric cancer database. Accordingly, renal cell carcinoma was diagnosed at a mean age of 64 (median 67, 41-78) years, compared with 62 years (median 63, range 9-88) in the renal cell carcinoma database. In five patients renal cell carcinoma was diagnosed prior to gastric cancer, in another five patients synchronous to gastric cancer, and in the remaining two patients after gastric cancer, respectively.

3.1.2 Additional tumors

In addition to gastric cancers and renal cell carcinomas, some of the patients developed further tumors: One (male) patient developed two colorectal adenocarcinomas, one in the rectum and two years later one in the descending colon, both with a mucinous component covering less than 50% of the tumor area. One (female) patient developed a colorectal adenocarcinoma and a renal oncocytoma, while another (female) patient had a ductal breast cancer (compare Table 4).

3.1.3 Follow-up

At time of last follow-up, only one patient was free of disease, and one other had died of myocardial infarction with no evidence of disease at time of death. Nine

patients experienced progression of malignant disease during follow-up, six of which were alive with disease and three had died of cancer at time of last follow-up. The remaining patient was diagnosed with hepatic metastases at time of diagnosis of gastric cancer; no further follow-up data were available. In general, no histology was obtained from metastatic tumor deposits (details are given in Table 4).

Table 4: Patient and tumor characteristics

No.	Sex	Renal Cell Carcinoma					Gastric Cancer					Outcome
		Age (yrs)	pT / pN	G	Histo type	Size (cm)	Age (yrs)	pT / pN	G	Histology / Lauren Classification	Location / Size (cm)	
#1	M	64	1B / X	2	Clear cell	5.5	70	X / X	3	Signet ring cell with mucinous component / diffuse	Cardia / X	AWD (peritoneal metastases) 2 months after diagnosis of gastric cancer (no tumor resection)
#2	M	57	1A / X	2	Clear cell	2	63	3 / 1	4	Undifferentiated / diffuse	Antrum / 5.5	DOD 10 months after diagnosis of gastric cancer
#3	F	78	1A / X	2	Clear cell	3	78	3 / 1	3	Signet ring cell / diffuse	Corpus / 2.2	Synchronous CRC (T3 N0) and renal oncocytoma, DOD 3 yrs after tumor diagnosis
#4	M	68	1A / X	1	Clear cell	1.2	61	2 / 1	3	Signet ring cell with mucinous component / diffuse	Corpus / 3	2 metachronous CRC, AWD (hepatic, pulmonal, and bone metastases) 10 yrs after diagnosis of gastric cancer
#5	F	66	1A / X	1	Clear cell	3	67	2 / 2	2	Tubular with mucinous component / intestinal	Cardia / 6	BC 4 yrs prior to RCC, AWD (bone metastases) 8 yrs after diagnosis of BC
#6	M	75	3A / X	2	Clear cell	7	75	3 / 2	3	Tubular / intestinal	Antrum / 6	AWD (local recurrence of gastric cancer) 8 months after tumor diagnosis

Table continues next page

No.	Sex	Renal Cell Carcinoma					Gastric Cancer					Outcome
		Age (yrs)	pT / pN	G	Histo type	Size (cm)	Age (yrs)	pT / pN	G	Histology / Lauren Classification	Location / Size (cm)	
#7	M	69	1A / X	2	Clear cell	3.5	68	3 / 0	4	Tubular with undifferentiated component / mixed	Gastric stump / 4.5	NED 10 yrs after diagnosis of gastric cancer
#8	M	45	1A / X	2	Clear cell	4	55	X / X	4	Undifferentiated / diffuse	Fundus / X	Hepatic metastases at time of diagnosis of gastric cancer, no follow-up
#9	M	75	1A / X	2	Clear cell	3.2	75	3 / 2	3	Signet ring cell / diffuse	Corpus / 6	DOC (myocardial infarction) 1 yr after tumor diagnosis
#10	M	41	1A / X	1	Clear cell	4	41	3 / 3	3	Signet ring cell with mucinous component / diffuse	Corpus / 9	DOD 2 yrs after tumor diagnosis of gastric cancer
#11	F	55	1A / X	1	Clear cell	3.5	62	3 / 3	3	Adenosquamous / mixed	Antrum / 7.6	AWD (hepatic, peritoneal, and bone metastases) 4 yrs after diagnosis of gastric cancer
#12	F	78	3A / X	3	Clear cell	4.5	78	1a / X	1	Papillary / intestinal	Antrum / X	AWD (pulmonal, bone, hepatic and pancreas metastases) 1 yr after tumor diagnosis

AWD, alive with disease; BC, breast cancer; CRC, colorectal cancer; DOC, died of other causes; DOD, died of disease; F, female; G, grade; M, male; NED, no evidence of disease; yrs, years

3.2 Histology

Details about the histology of all tumors including T and N classification, tumor size and location, as well as Laurén's classification for gastric cancer are presented in Table 4.

3.2.1 Renal cell carcinomas

All renal cell carcinomas were of clear cell type (Figure 7). In general, they were well differentiated and were predominantly diagnosed at an early tumor stage.

Regarding T-classification (47), ten out of twelve cases were pT1, which means the tumor was limited to the kidney and had a diameter of 7 cm or less in greatest dimension. Nine of them were pT1A, having a tumor diameter of 4 cm or less in greatest dimension and one of them was pT1B with a tumor diameter of 7 cm or less in greatest dimension. Two of the patients had pT3a tumors (#6,#12) characterized by invasion of perirenal fat.

None of the renal cell carcinomas had lymph node metastases.

Regarding tumor grade (2), eleven of twelve patients had G1 or G2 tumors, one (patient #12) had a G3 tumor.

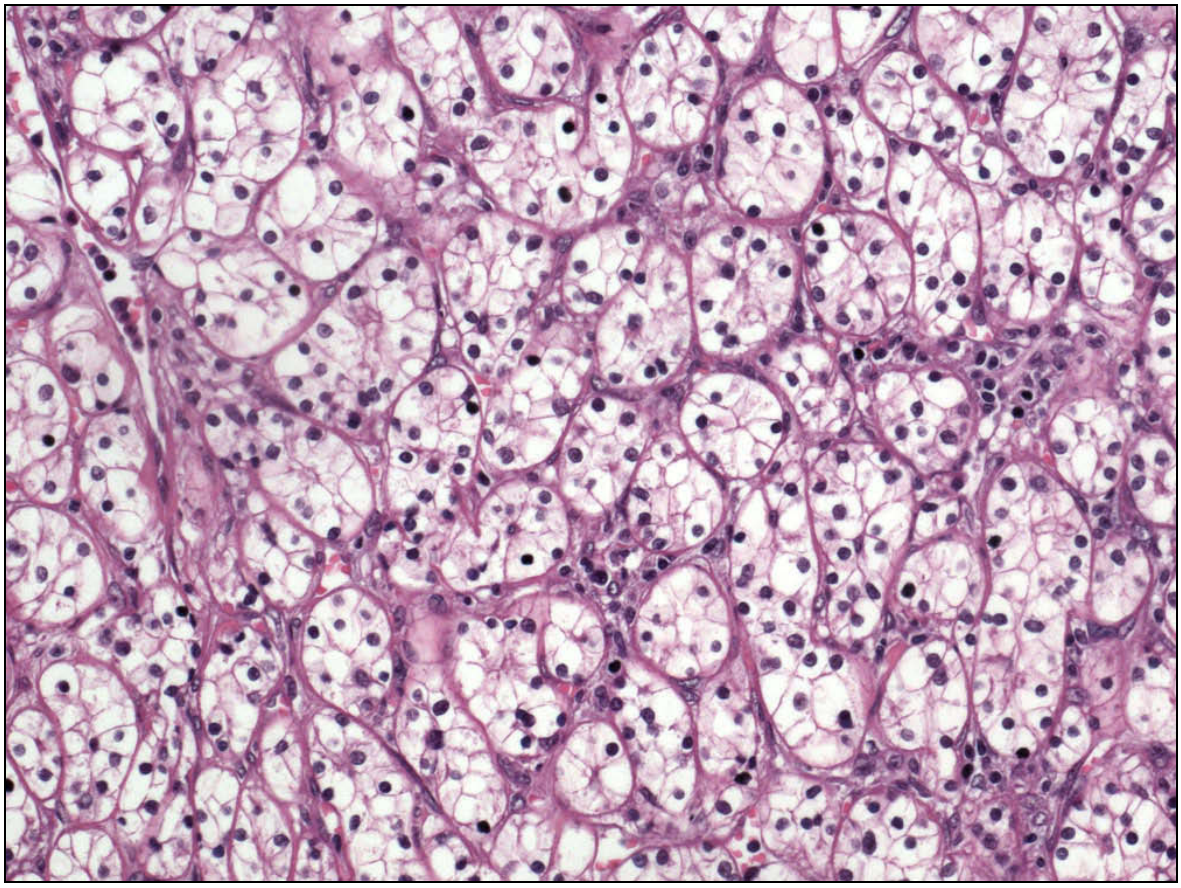

Figure 7: Clear cell renal carcinoma of patient #5 (H&E, original x100).

The tumor is well differentiated. It is well representative for most of the renal carcinomas of our patients, since all of them were clear cell carcinomas and eleven of twelve were Grade 1 or 2. Overall, this tumor was G1 according to WHO classification and pT1A according to AJCC-UICC. The patient had T1, G1, N0 ductal breast cancer at the age of 62. Four years later, she developed the renal cell carcinoma displayed above, and within one year was also diagnosed with gastric cancer. Later in the disease course, bone metastases thought to be due to her breast cancer were found.

3.2.2 Gastric cancers

Gastric tumors were poorly differentiated or even undifferentiated in the vast majority of cases (10 out of 12) and were generally diagnosed at a high tumor stage.

Five tumors were diagnosed as signet ring cell carcinomas (patients #1, #3, #4, #9, #10) of which three showed extracellular mucin production (#1, #4, #10; Figure 8).

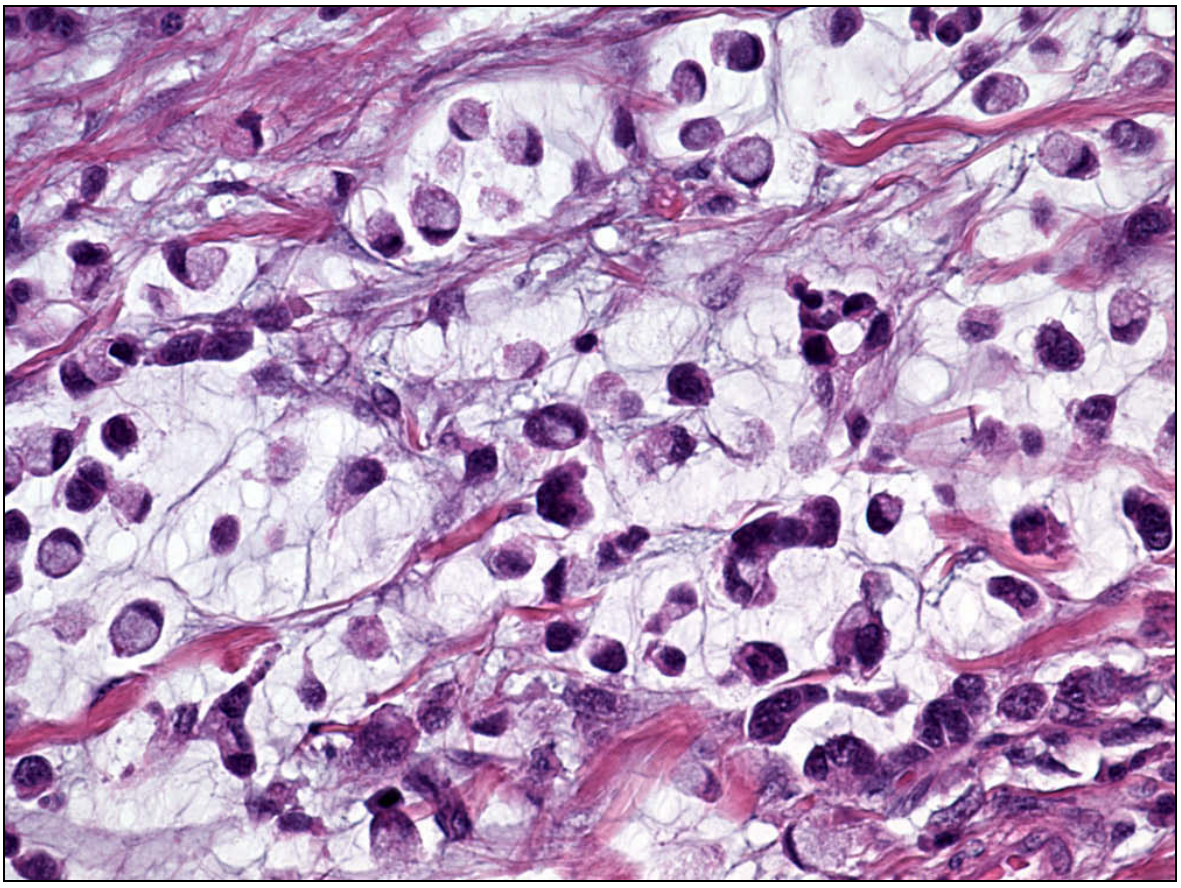

Figure 8: Gastric signet ring cell carcinoma with extracellular mucin production obtained from the resection specimen of patient #10 (H&E, orignial x100).

The patient had synchronous renal cell carcinoma (pT1A G1) at the age of 41. The pathologic evaluation of the resection specimen of his gastric cancer had also revealed metastatic spread to 24 out of 35 lymph nodes (N3). He experienced local tumor recurrence and died of disease two years after the initial diagnosis.

One patient had a moderately differentiated tubular adenocarcinoma with a mucinous component (#5), one patient an adenosquamous (#11, Figure 9) and an-

other patient a well differentiated papillary (#12) carcinoma. Three tumors were diagnosed as undifferentiated carcinomas (#2, #7, #8) of which one showed areas of a pre-existing moderately differentiated tubular adenocarcinoma (#7). The undifferentiated carcinoma of patient #2 showed large areas of necrosis and was found to be densely infiltrated by mixed inflammatory cells (Figure 10). The undifferentiated carcinoma with pre-existing better differentiated areas of patient #7 was characterized by a dense intra- and peritumoral lymphoplasmacellular infiltrate including lymph follicle formation (Figure 11).

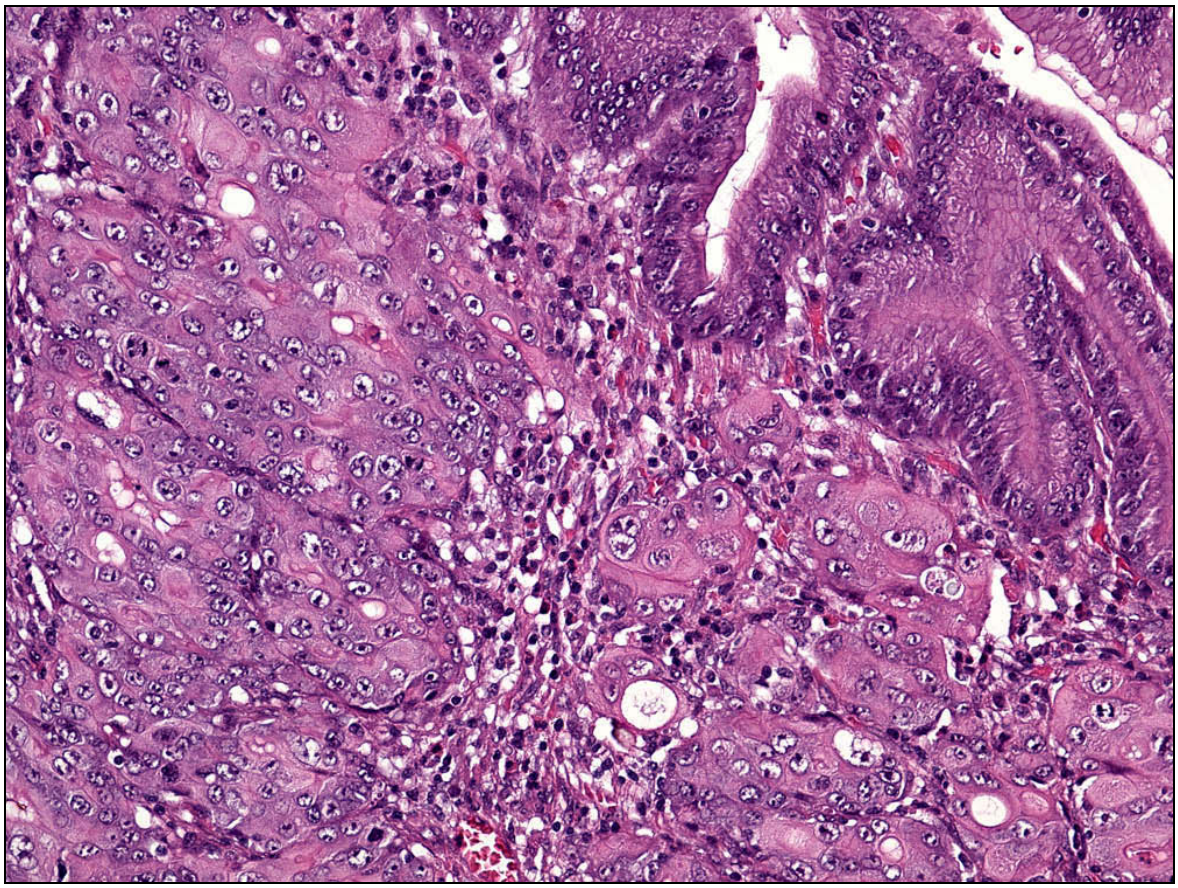

Figure 9: Poorly differentiated adenosquamous gastric carcinoma of patient #11 (H&E, original x100).

The picture shows poorly differentiated solid and squamous tumor growth. The lesion measured 7.2 cm in largest diameter and was located in the antrum. The patient developed bone, hepatic and peritoneal metastases. She had had clear cell renal cell carcinoma (pT1A G1) seven years before diagnosis of gastric cancer.

Regarding T classification, seven patients had pT3 gastric cancers, meaning the tumors were growing into the subserosa, two tumors (patients #4 and #5) were classified pT2, which stands for invasion of tumor cells into the muscularis propria. One tumor (#12) was classified pT1a (tumor growing into the lamina propria or muscularis mucosae). The two remaining gastric cancers were pTx, because only biopsy material was available from these patients (#1,#8).

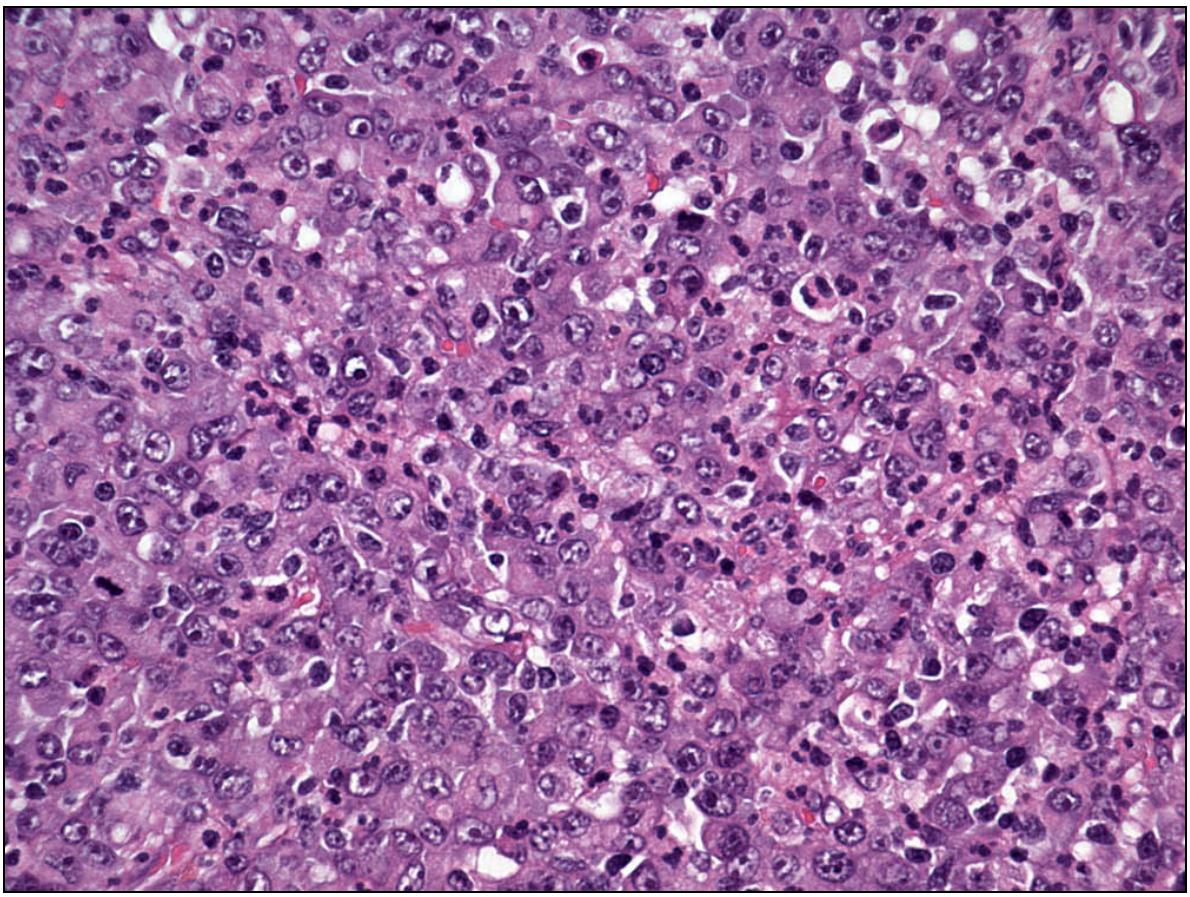

Figure 10: Undifferentiated gastric carcinoma of patient #2 with marked mixed intratumoral inflammation (H&E, original x100).

The resection specimen showed an exulcerated tumor that infiltrated into the serosa and the lesser omentum. Two of the resected lymph nodes had metastases. The patient had had renal cell carcinoma 6 years prior to diagnosis of gastric cancer. Immunohistochemistry of the gastric cancer tissue showed loss of mismatch repair proteins hMLH1 and PMS2 (compare Figure 18). Molecular analysis of this cancer specimen revealed a high degree of microsatellite instability (compare Figure 20) and also a *KRAS* mutation. The patient died 10 months after surgery.

Eight patients had lymph node metastasis from gastric cancer: Three (#2-4) were classified N1 (metastasis in 1 to 2 regional lymph nodes), three (#5,#6,#9) were N2 (metastasis in 3-6 regional lymph nodes) and two (#10,#11) were N3 (metastasis in 7 or more regional lymph nodes). Only one patient had no regional lymph node metastasis (#7), while for another three patients (#1,#8,#12) regional lymph nodes could not be assessed.

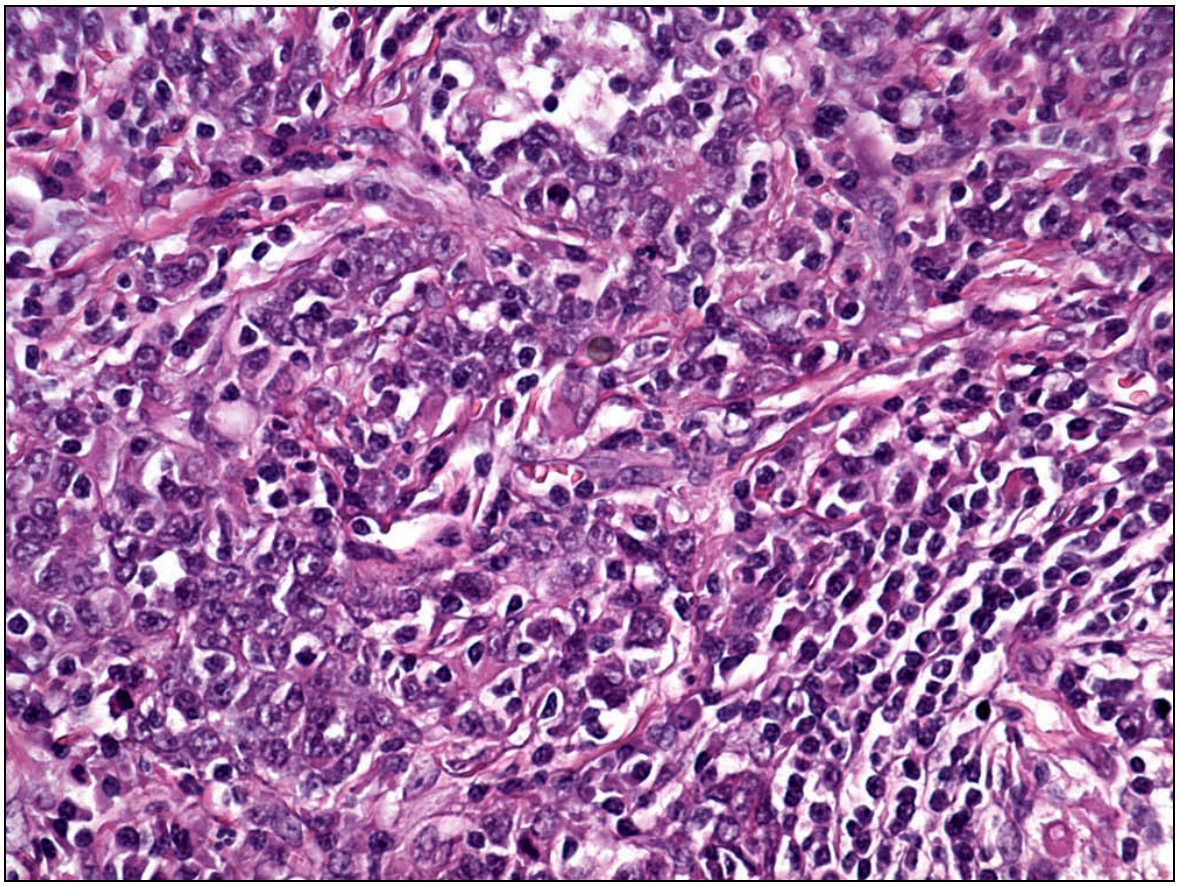

Figure 11: Undifferentiated component of gastric carcinoma with marked lymphoplasmacellular inflammation of patient #7 (H&E, original x100).

The patient developed gastric cancer aged 68 in gastric stump after Billroth II operation. Our study could relate the peculiar morphology to EBV infection (compare below and Figure 19). The patient was diagnosed renal cell carcinoma one year later and was treated by nephrectomy.

3.2.3 Colorectal cancers

The colorectal cancer of patient #3 was a poorly differentiated adenosquamous carcinoma with a mucinous component (<50% of the tumor area, Figure 12), while both colorectal cancers of patient #4 were poorly differentiated adenocarcinomas with a mucinous component (<50% of the tumor area, Figure 13).

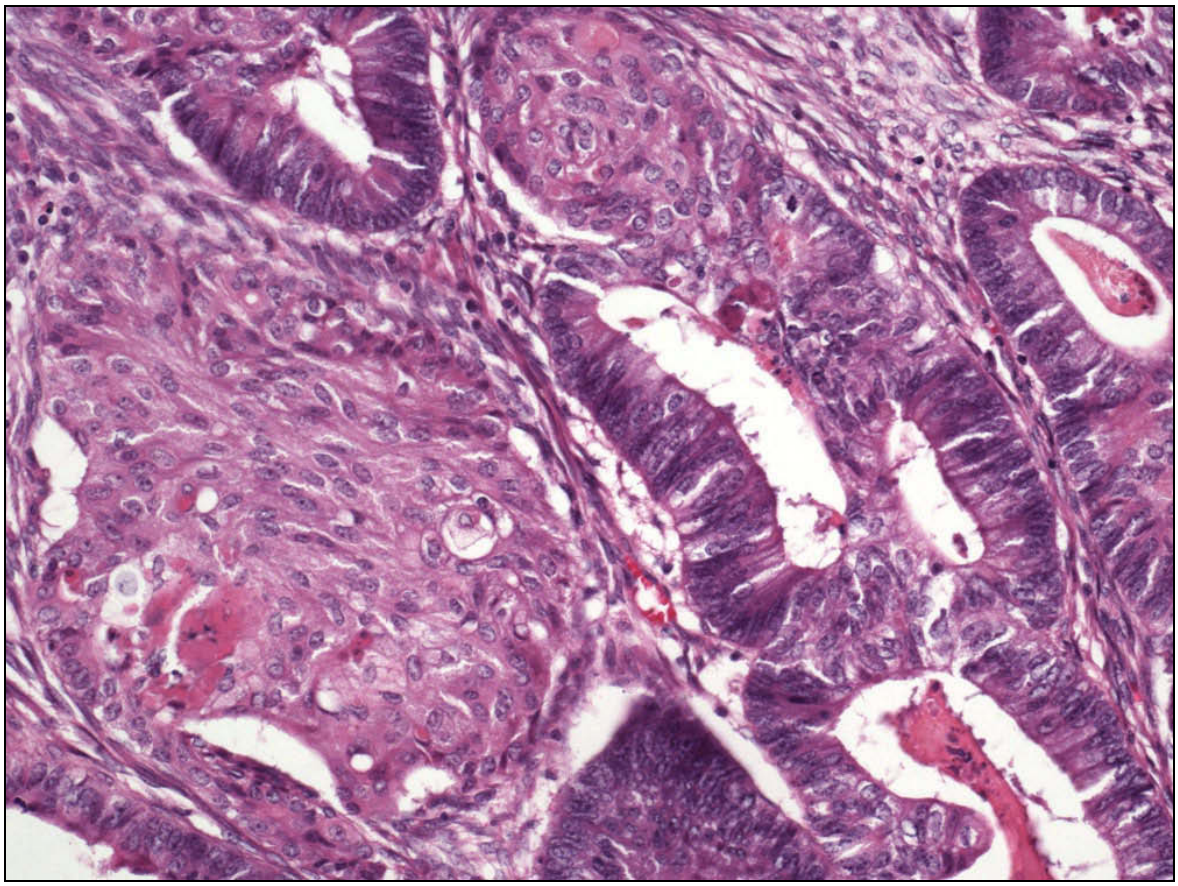

Figure 12: Adenosquamous colorectal carcinoma of patient #3 (H&E, original x100).

The image shows an area with both squamoid and glandular differentiation. The patient was diagnosed with gastric cancer, synchronous renal cell carcinoma and synchronous colorectal cancer at age 78. Moreover, the patient had a renal oncocytoma. She died of disease three years after diagnosis of her tumors.

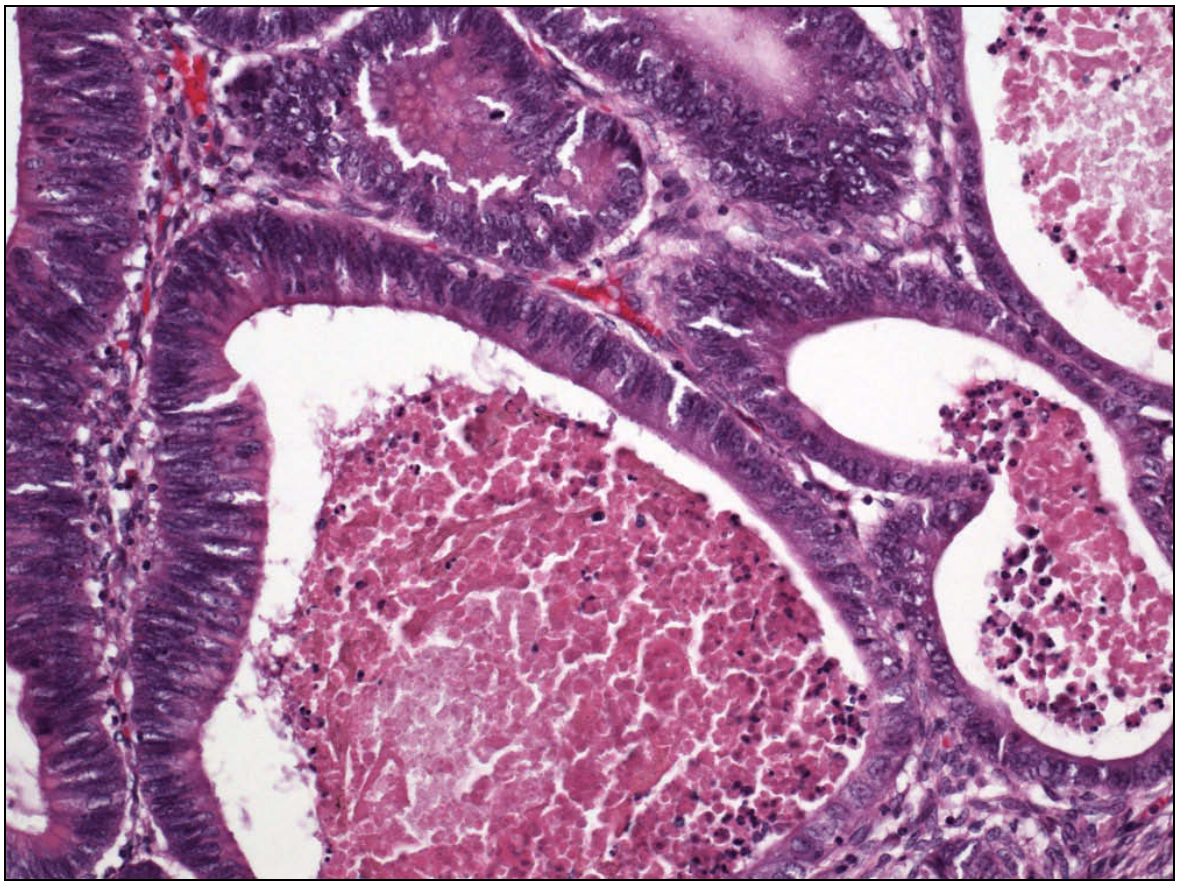

Figure 13: The first of two colorectal adenocarcinomas of patient #4 (H&E, original x100).

The image shows neoplastic glands with necrotic detritus within the lumen. The patient underwent gastrectomy for pT2, N1, G3 gastric cancer at age 61. Seven years later, he was diagnosed rectal cancer (displayed above) and clear cell renal cell carcinoma. Another two years later, a second colorectal cancer was found in his descending colon / sigmoid colon. He developed pulmonary, hepatic and bone metastases before he was lost to follow-up.

3.3 Immunohistochemistry

Immunohistochemical (and molecular) findings in gastric cancers are illustrated in Table 5 (p.54).

High (3+) proliferative activity (MIB-1) was present in the majority of gastric cancer cases (Figure 14). P53 protein overexpression was observed in seven car-

cinomas and varying loss and/or reduction of membranous E-cadherin immunolabeling in ten carcinomas (Figures 15,16). Two gastric cancers (patients #2 and #11) showed loss of nuclear expression of mismatch repair proteins hMLH1 and PMS2 (Figures 17,18). This finding was confirmed by molecular analysis showing a high degree of MSI (compare below).

All renal cell carcinomas as well as the three colorectal cancers from patients #3 and #4 retained mismatch repair protein expression.

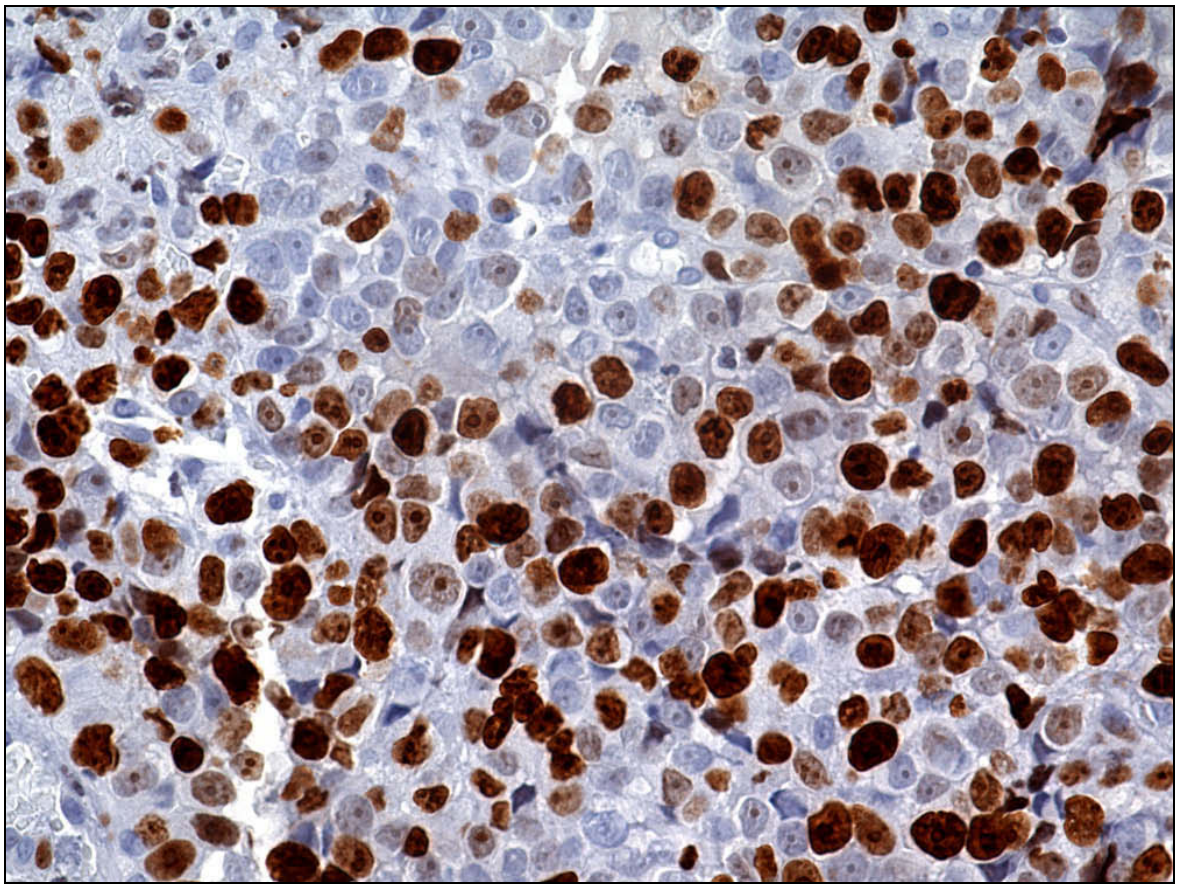

Figure 14: Undifferentiated gastric cancer of patient #8: Immunohistochemistry reveals high proliferative activity as demonstrated by MIB-1 (ki-67) immunolabeling (original x100).

This patient had been operated for renal cell carcinoma at the age of 45, which was ten years before diagnosis of gastric cancer. At the time of diagnosis of gastric cancer, he had hepatic metastases, so he did not undergo gastrectomy, but was treated with palliative care.

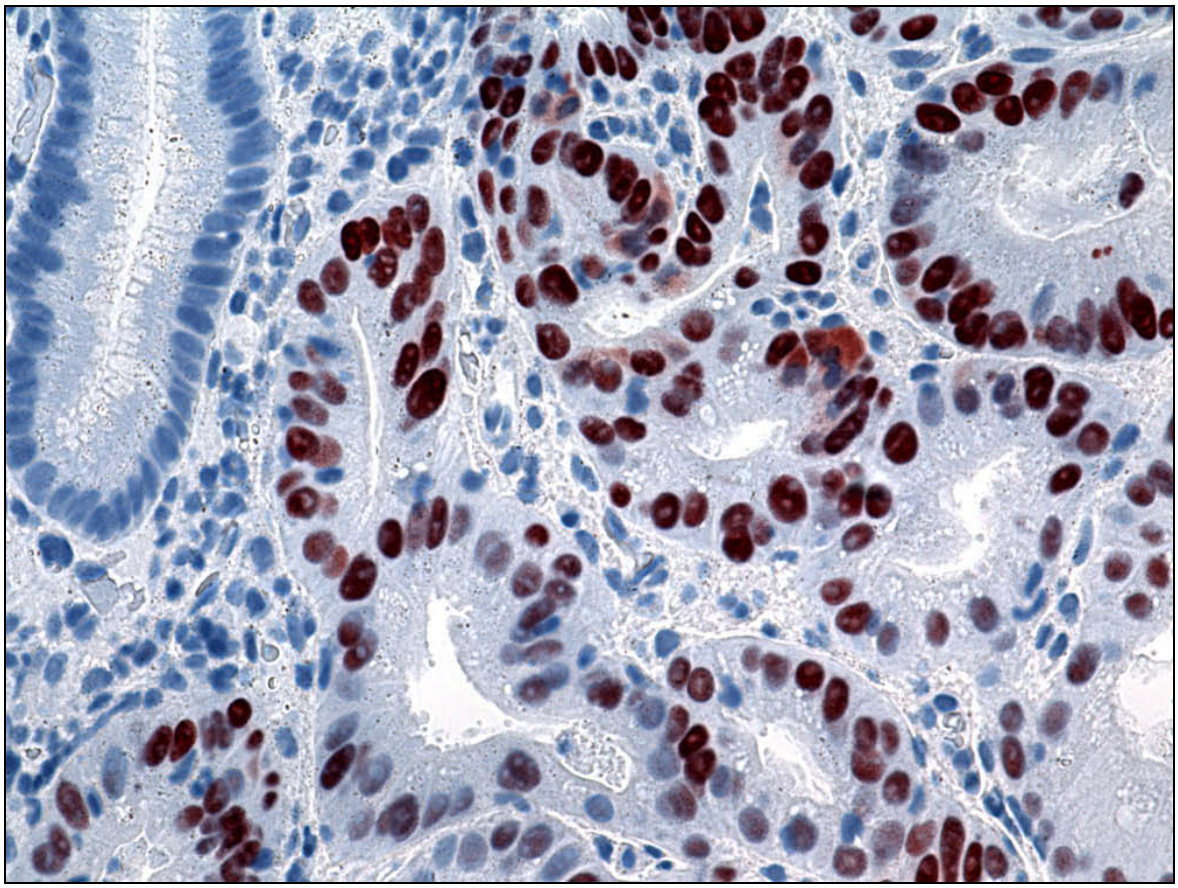

Figure 15: p53 overexpression of gastric cancer cells in patient #5 (original x100).

The p53 positive cells show intensive brown nuclear staining. The patient had T1, G1, N0 ductal breast cancer at the age of 62. Four years later, she developed renal cell carcinoma (compare Figure 7), and within one year was also diagnosed with gastric cancer with lymph node metastasis. Furthermore, bone metastases thought to be due to her breast cancer were found.

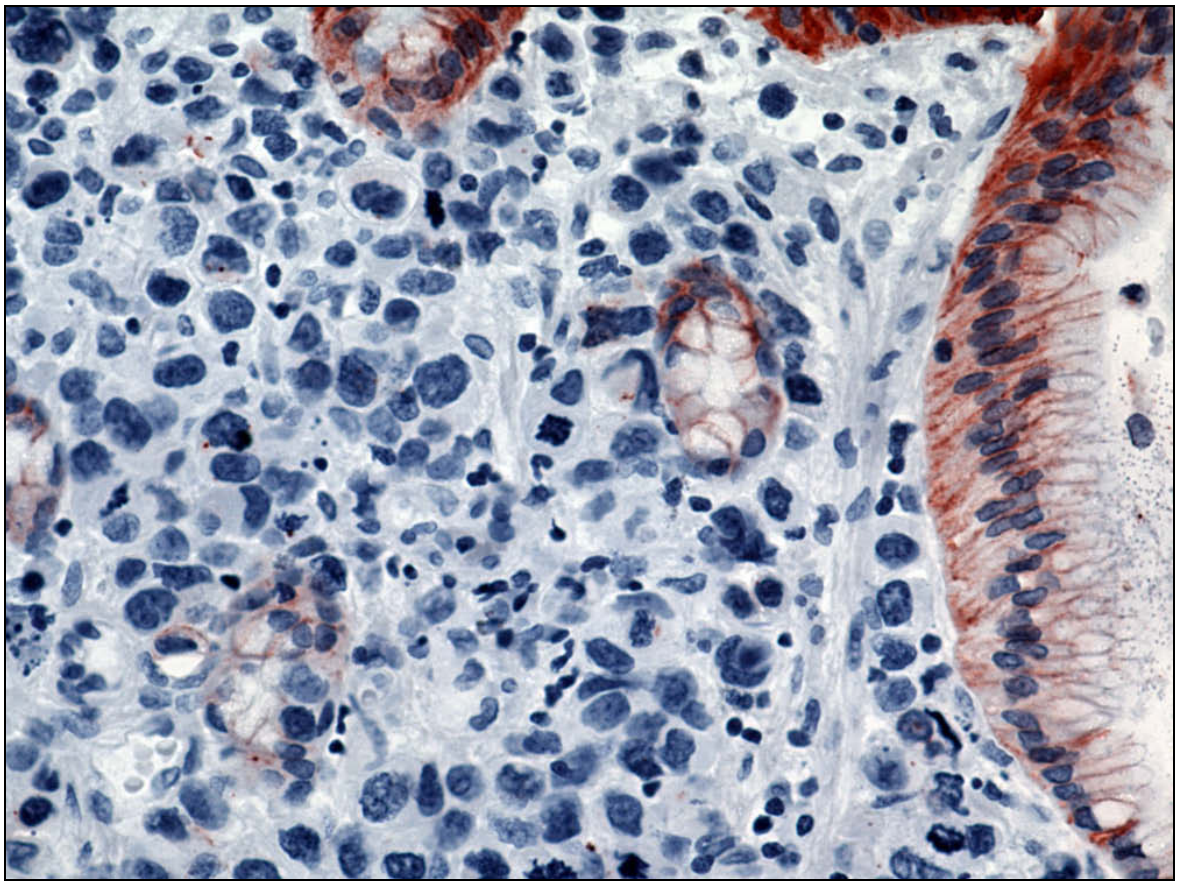

Figure 16: Loss of E-cadherin staining in poorly differentiated gastric cancer in patient #9 (original x100).

The image also shows non-neoplastic gastric foveolae (right) specifically labeled by the anti E-cadherin antibody (positive internal control). The patient was 75 years old when he underwent gastrectomy for this gastric cancer classified pT3 G3 N2 and nephrectomy for his synchronous pT1A G2 renal cell carcinoma. Two days after surgery, he experienced myocardial infarction and one year later died of re-infarction.

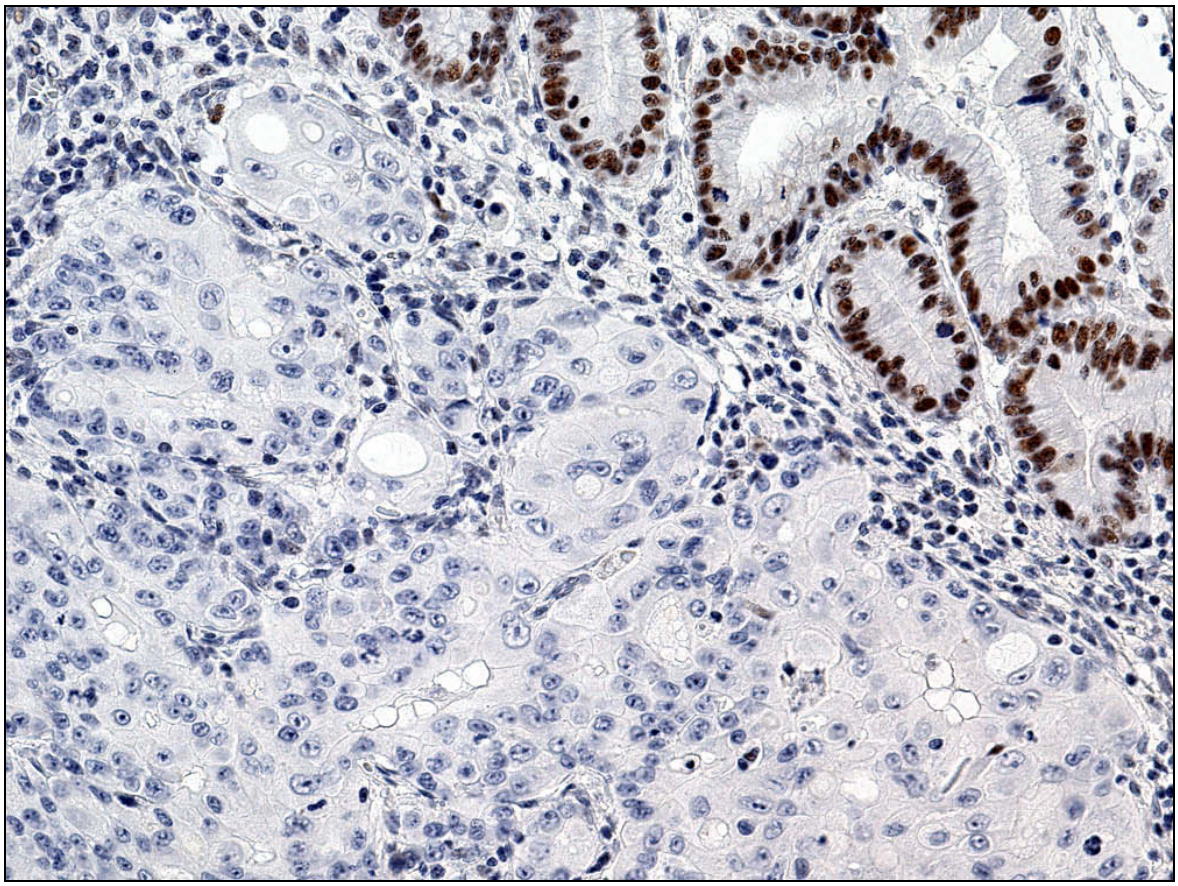

Figure 17: Loss of hMLH1 immunostaining in adenosquamous gastric carcinoma of patient #11 (original x100).

On the right upper corner normal gastric mucosa is visible, which shows positive nuclear immunolabeling with hMLH1 antibody (internal positive control). The tumor was also negative for PMS2 and showed a high degree of microsatellite instability on molecular analysis. Compare Figure 9 for H&E of this tumor.

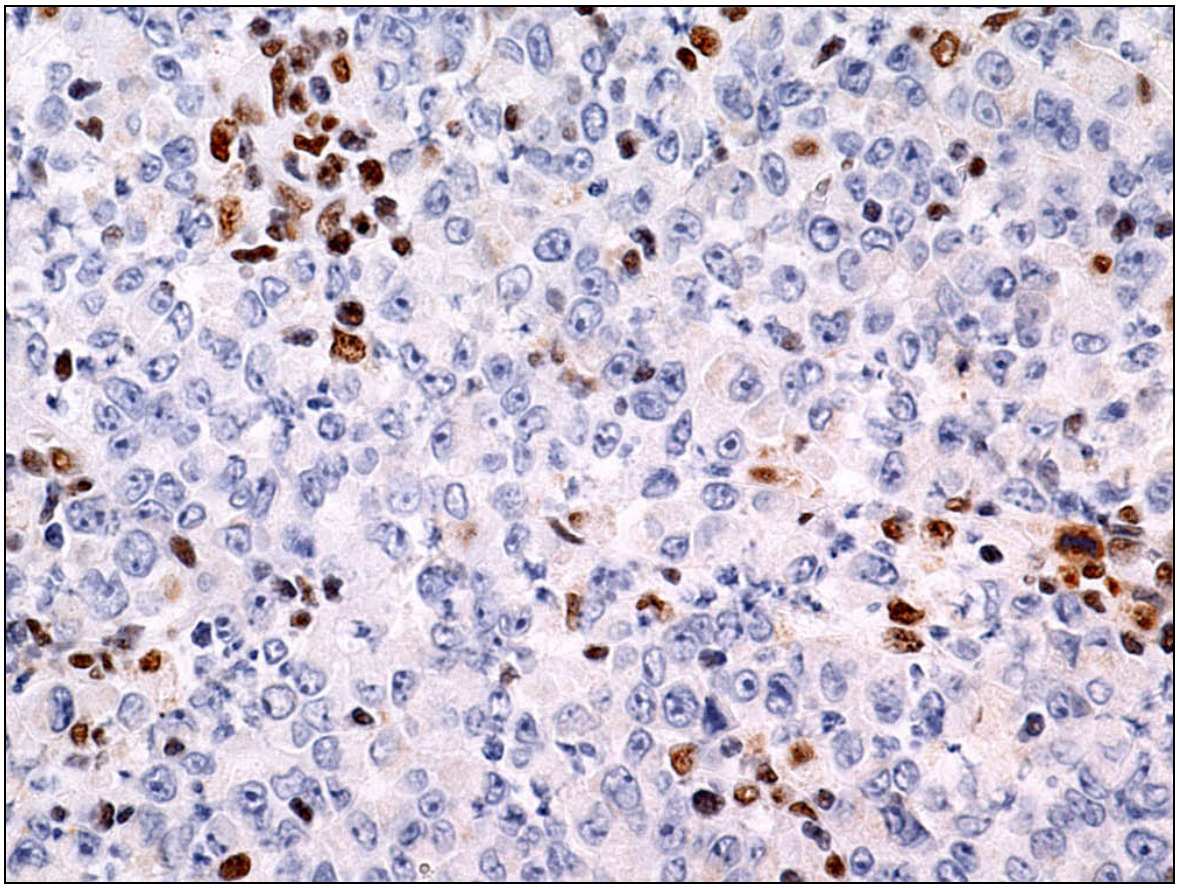

Figure 18: Loss of PMS2 immunostaining in undifferentiated gastric tumor tissue obtained from patient #2 (original x100).

Note distinct nuclear staining of intratumoral inflammatory cells (internal positive control), while all cancer cells are negative. Immunohistochemistry also showed loss of mismatch repair protein hMLH1. Molecular analysis of this cancer specimen revealed a high degree of microsatellite instability and also a *KRAS* mutation. Compare Figure 10 for H&E of this tumor and Figure 20 for MSI testing results.

Table 5: Immunohistochemical and molecular findings in gastric cancers

Patient	Immunohistochemistry							Molecular Analysis / ISH		
	p53	MIB1	E-cad	MLH1	MSH2	MSH6	PMS2	MSI	*KRAS*	EBER
#1	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
#2	-	+++	pos	neg	pos	pos	neg	MSI-H	mut	neg
#3	-	+	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
#4	-	+++	↓↓	pos	pos	pos	pos	MSS	wt	neg
#5	++	+	↓	pos	pos	pos	pos	MSS	wt	neg
#6	-	++	↓	pos	pos	pos	pos	MSS	wt	neg
#7	+++	+++	↓	pos	pos	pos	pos	MSS	wt	pos
#8	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	u	neg
#9	+++	+	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
#10	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
#11	-	+	↓	neg	pos	pos	neg	MSI-H	wt	neg
#12	+++	+++	pos	pos	pos	pos	pos	MSS	wt	neg

E-cad, E-cadherin; ISH, *in situ* hybridisation; MSI-H, microsatellite instability-high; MSS, microsatellite stable; mut, mutated; neg, negative (or ↓↓↓ for E-cadherin); pos, positive; wt, wild type; u, unknown; +, weak; ++, moderate; +++, extensive; ↓, reduced; ↓↓, markedly reduced

3.4 In situ hybridization

Presence of EBV was demonstrated in the gastric cancer characterized by dense intratumoral lymphoplasmacellular infiltrate in H&E stained sections (Patient #7), whereas all other tumors were negative (Figure 19).

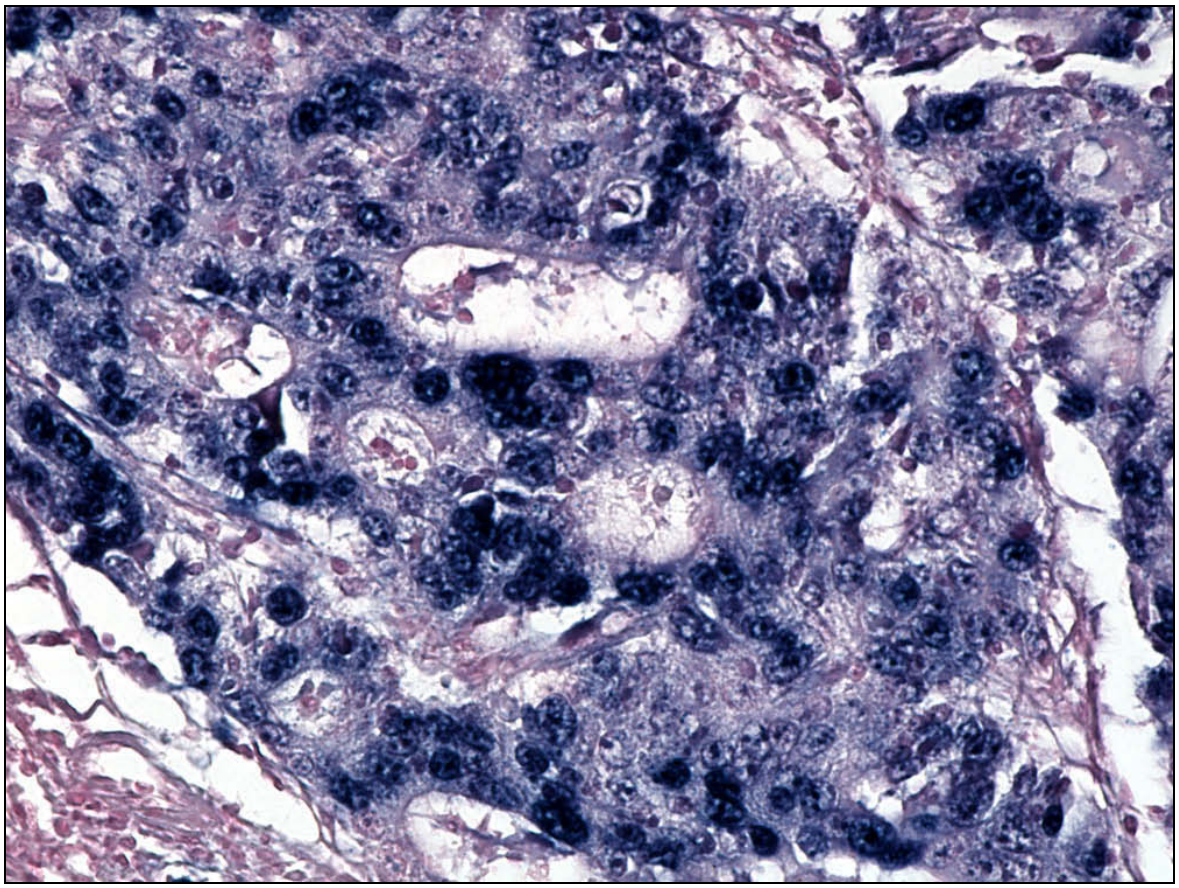

Figure 19: EBV *in situ* hybridization (EBER) of gastric tumor tissue obtained from patient #7 (original x100).

The patient had a tubular gastric carcinoma with undifferentiated component and marked intratumoral lymphoplasmacellular inflammation (compare Figure 11 for H&E).

3.5 Microsatellite analysis

Testing for MSI, we found additional peaks for the microsatellite markers NR-21, BAT-26, BAT-25, NR-24 and MONO-27 in the gastric cancer of patient #2, compared with the patient's normal and renal cancer tissue (Figure 20). In patient #11 we observed a characteristic peak broadening regarding the markers NR-21, BAT-26, BAT-25 and MONO-27 in gastric cancer tissue, compared with the patient's normal and renal cancer tissue. Hence, we classified both patients MSI-high, which is concordant with the immunohistochemical findings in these patients.

We did not find MSI in any of the renal cell carcinomas. The colorectal cancers of the patients #3 and #4 were also MSS.

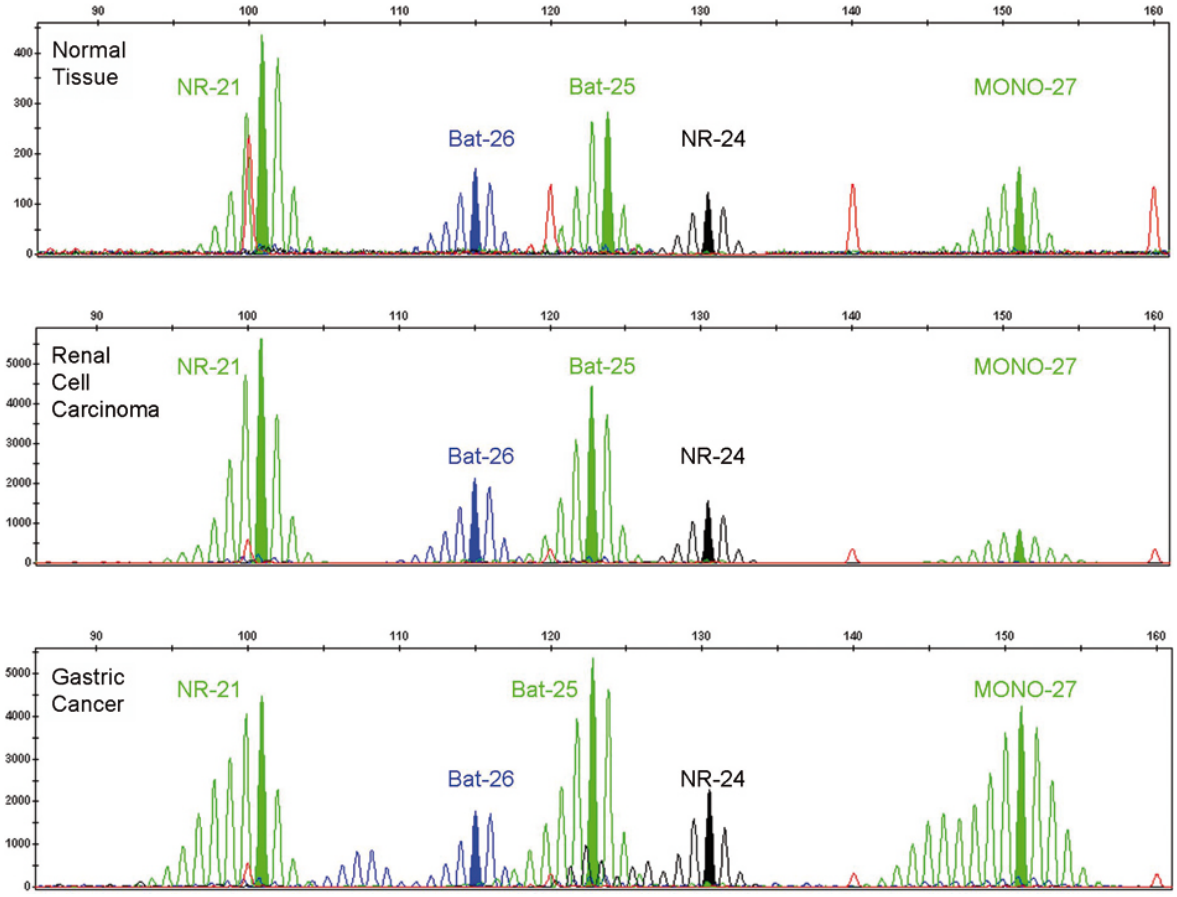

Figure 20: MSI testing shows additional peaks for the microsatellite markers NR-21, BAT-26, BAT-25, NR-24 and MONO-27 in the gastric cancer of patient # 2 (below), but not in the patient's normal tissue or renal cancer tissue.

3.6 KRAS mutation analysis

Screening for *KRAS* mutations in codons 12 and 13 was positive in one of the gastric cancers: We found a transversion form guanine into cytosine at the second base of codon 12 (GGT → GCT) resulting in Gly12Ala in the gastric cancer of patient #2. Additionally we found *KRAS* mutations at the second base of codon 12 in the colorectal cancers: The colon cancer of patient #3 had a transversion into thymine (GGT→ GTT; Gly12Val) and the rectum cancer of patient #4 a transition into adenine (GGT→ GAT; Gly12Asp) (Figure 21). We did not detect *KRAS* mutations in any of the renal cell carcinomas. The material of one gastric cancer could not be analyzed because of poor DNA quality.

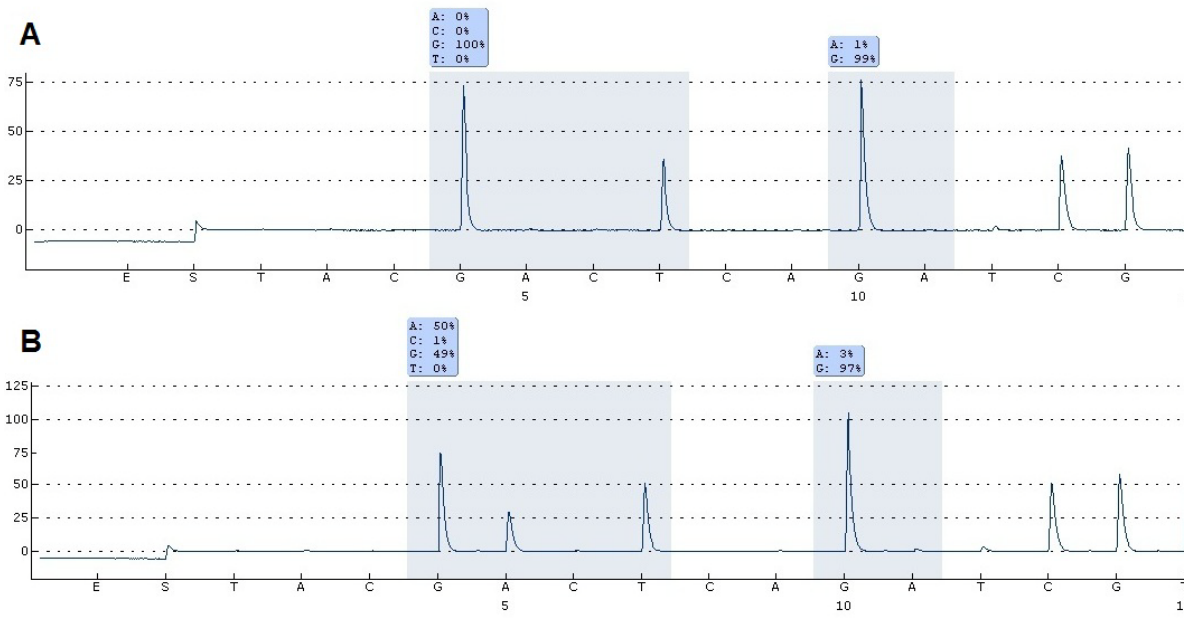

Figure 21: Pyrosequencing shows a *KRAS* mutation in the rectum cancer (B) of patitent #4, but not in gastric cancer of the same patient (A).

The analysis of DNA from the gastric cancer shows the expected "double peak" for guanine and a normal height peak for thymine at codon 12 (left area highlighted blue), which represents the wild type "GGT". The rectum cancer has a smaller guanine peak and an additional adenine peak, which points to the mutated genotype "GAT" in a large amount of analyzed cells. Codon 13 (right area highlighted blue) shows wild type "GGC" in both tumor probes displayed.

4 Discussion

Carcinogenesis is driven by progressive accumulation of somatic mutations in a number of tumor related genes. Patients with multiple neoplasms are more likely to harbor a germline mutation in one of those genes. It is estimated that approximately 5% of all cancers arise within a hereditary cancer syndrome, each of which presenting with a spectrum of tumors (52).

A systematic study analyzing the association of gastric and renal cancer is lacking. The present study focused on twelve patients with gastric and syn- and/or metachronous renal cell carcinoma based on the observation of a remarkably high incidence of gastric cancer in patients with renal cell carcinoma, suggesting the possibility of a hitherto unrecognized etiological association or hereditary cancer syndrome.

Manifestation age regarding both gastric and renal cancer, however, was not significantly different from that of patients included in the gastric and renal cancer databases, respectively. Moreover, the male predominance of our cohort is in the line with epidemiologic data from our country, where the risk for male patients to develop gastric or renal cancer is almost twice as high as for females (45). International data show a similar distribution between the sexes (12).

All renal cell carcinomas were of clear cell type, which is known to represent the most common form of renal cancer worldwide. Nevertheless, it was noteworthy that eleven of the twelve tumors were well differentiated (grade 1 or 2), while the majority of gastric cancers were very poorly differentiated (grade 3) or even undifferentiated (grade 4) at the time of diagnosis, except for patient #12, who had a well differentiated gastric cancer and a grade 3 renal cell carcinoma.

Some of the poorly differentiated gastric tumors harbored marked intratumoral inflammation or presented with rather uncommon histological subtypes, such as

adenosquamous carcinoma. According to literature data, intratumoral mixed or predominantly lymphoplasmacellular infiltration has been related to MSI or EBV infection (53-56), respectively.

This led to the hypothesis, that Lynch syndrome may have caused gastric cancer associated with renal cancer in our cohort. In addition, we searched for EBV in cancer tissues, particularly since EBV is associated with hypermethylation of tumor suppressor genes known to be involved gastric cancer (57). Entities for which EBV detection is of diagnostic value are summarized in Table 6 (49).

Table 6: Entities for which Epstein–Barr virus (EBV) detection is of diagnostic value (49)

Entity	**EBER expression (%)**
Infectious mononucleosis	100
Smooth muscle tumour	100
Gastric lymphoepithelial carcinoma	100
Gastric adenocarcinoma	5–15
Hodgkin lymphoma	40–60
Lymphomatoid granulomatosis (B type)	90
Angioimmunoblastic T cell lymphoma	81–95
Richter syndrome	15–20
NK leukaemia	100
Nasal type T/NK	100
Liver follicular dendritic cells	100

EBER, Epstein Barr encoded RNA

We found the gastric cancer with marked mixed infiltrate to be MSI (Patient #2) and the gastric cancer with predominantly lymphoplasmacellular infiltrate to be associated with EBV infection (#7). The second gastric cancer with MSI showed adenosquamous differentiation (#11) which has been related to MSI-high status in 12% (2 out of 17) of cases (58). However, the overall incidence of MSI-high status (two out of twelve; 17%) in our cohort of gastric cancers is in the line with literature data ranging from 10 to 20% of tumors (59-63). All renal cancers analyzed in our study were MSS. Data on the presence of MSI or mismatch repair gene defects in renal cell carcinomas are conflicting. Reported incidences range from 0 to 40% (64-72). The fact that, in our series, both renal tumors from patients with MSI-high gastric cancers were MSS argues against germline mutations in mismatch repair genes (Lynch syndrome) and we conclude that neither Lynch syndrome nor EBV infection represents a major cause with respect to carcinogenesis in our patient cohort.

A crucial pathway involved in cell proliferation is the *RAS–RAF–MEK–ERK–MAP* kinase pathway. In fact, *RAS* genes are reported to be the most frequently mutated oncogenes in human cancers. Hotspot mutations in codons 12, 13 or 61 lead to activation by fixing the Ras protein in GTP-bound, active state. (73,74).

In contrast to colorectal cancer, *BRAF* mutations are exceedingly rare in gastric cancers and do not characterize MSI tumors (75). Frequencies of *KRAS* mutations in gastric cancer of up to 8.5% have been reported (11,58,76-80). In renal cell carcinomas the incidence of *KRAS* mutations is lower (81), while activating mutations in the *KRAS* gene are found in 30-40% of colorectal carcinomas (82). Patient #2 had a gastric cancer harboring a *KRAS* mutation and was also MSI. Additionally, we observed *KRAS* mutations in two patient's colorectal carcinomas, but in none of the renal cancers. Hence, the observed frequencies in our patients are in agreement with the literature. In addition, data suggest that *KRAS* mutations are more frequent in MSI-high gastric cancers than in MSS. Thus, Brennetot et al. (83) observed *KRAS* mutations in 28% of MSI gastric cancer, while no mutations were noted in MSS tumors.

Aditionally, there are some hereditary renal tumor syndromes (Table 7), some of these may cause clear cell renal cell carcinoma, but none of these is related to a higher frequency of gastric cancer. Most common is von Hippel-Lindau disease, which leads to (often multifocal) clear cell renal cell carcinoma and renal cysts and is additionally associated with CNS- and retinal haemangioblastomas, pheochromocytomas, pancreatic cysts and neuroendocrine tumors as well as endolymphatic sac tumors of the inner ear, epididymal and broad ligament cystadenomas (84). The Birt-Hogg-Dubé syndrome also includes clear cell renal cell carcinoma or oncocytoma, but most often chromophobe- or oncocytic hybrid tumors as renal manifestation, while fibrofolliculomas, trichodiscomas and acrochordons may be seen as extrarenal manifestations. Moreover, spontaneous pneumothoraces due to lung cysts are frequent in these patients (84). Finally, clear cell renal cell carcinoma may also occur in patients with constitutional chromosome 3 translocation, a very rare syndrome leading to multiple bilateral clear cell renal cell carcinomas without additional systemic manifestations (85). These syndromes, however, did not fit to the clinicopathological presentation of our patients.

Table 7: Major hereditary tumor syndromes involving the kidney (2,84-87).

Syndrome	Gene / location / function	Inh.	Renal manifestations	Other manifestations
Von Hippel-Lindau	*VHL* / 3p25 / TS	AD	Clear cell RCC (multiple and bilateral, solid and/or cystic), renal cysts	Retinal and CNS hemangioblastomas, pheochromocytoma, pancreatic cysts and neuroendocrine tumors, endolymphatic sac tumors of the inner ear, epididymal and broad-ligament cystadenomas
Hereditary papillary renal cancer	*cMET* / 7q31 / OG	AD	Papillary RCC type 1 (multiple and bilateral, solid)	None
Hereditary leiomyomatosis RCC	*FH* / 1q42-43 / TS	AD	Papillary RCC type 2, collecting duct carcinoma (solitary)	Uterine leiomyomas and leiomyosarcomas, cutaneous leiomyomas
Birt-Hogg-Dubé	*BHD* / 17p11.2 / TS	AD	Chromophobe RCC, clear cell RCC, papillary RCC, hybrid oncoytoma, oncoytic tumors (multiple)	Cutaneous / facial fibrofolliculomas, lung cysts, spontaneous pneumothorax, lipomas
Constitutional chromosome 3 translocation	unknown gene	AD	Clear cell RCC (multiple, bilateral)	None
Hyperparathyroidism jaw tumor	*HRPT2* / 1q25-32 / TS	AD	Papillary RCC, mixed epithelial and stromal tumors	Parathyroid tumors, fibro-osseous mandibular and maxillary lesions
Familial papillary thyroid cancer	unknown gene / 1q21		Papillary RCC, oncocytoma	Papillary thyroid cancer, nodular thyroid disease
Tuberous sclerosis complex	*TSC1* (Hamartin) / 9q34, *TSC2* (Tuberin) / 16p13.3 / TS	AD	Angiomyolipomas (multiple, bilateral), lymphangioleiomyomatosis, cysts.	Cutaneous angiofibroma (adenoma sebaceum), subungual fibromas, cardiac rhabdomyomas, adenomatous polyps of the duodenum and the small intestine, lung cysts, cortical tubers and subependymal giant cell astrocytomas

AD, autosomal dominant; CNS, central nervous system; FH, fumarate hydratase; HRPT2, hyperparathyroidism 2; Inh., inheritance; OG, oncogene; RCC, renal cell carcinoma; TS, tumor suppressor; VHL, von Hippel-Lindau

It is increasingly recognized, that patients who survived cancer are at higher risk for a second primary neoplasm, attributed not only to host characteristics like genetic susceptibility or hereditary factors, but also to environmental exposures and especially to prior cancer treatment (e.g. radiation-associated tumors or chemotherapy-induced leukemia), or to a combination of these influences (88). In our patients, radiation- or chemotherapy did not seem to play a major role. In five patients renal cancer was diagnosed synchronous with gastric cancer. In another five patients, renal cancer was diagnosed prior to gastric cancer, but all of these patients had stage I renal cell carcinoma. Thus, the patients were treated by partial, simple or radical nephrectomy, while no radiation or chemotherapy was given (89). In the remaining two patients (#4 and #7) renal cell carcinoma was diagnosed after gastric cancer. However, in patient #7 this was only one year later, and regarding patient #4, it is not clear whether he received chemotherapy or radiation for treatment of gastric cancer.

The fact that all of the investigated patients are from the region of Styria might suggest that founder effects resulting from genetic drift or environmental factors could have played a role.

It is also conceivable that multiple genes, i.e. polymorphisms, interacting with environmental factors and with each other, are the reason why our patients developed gastric cancer and concomitant renal cell carcinoma.

Regarding possible etiologic factors for the development of renal cell carcinoma and concomitant gastric cancer, however, environmental factors could not be analyzed here, due to the retrospective nature of our study and limited data in patient-charts and from attending physicians.

Also, specific data regarding the family history of the patients, which could have given additional hints regarding hereditary factors, were not available.

Regarding our molecular analysis, it has to be noted critically that the testing for MSI, *KRAS* mutations or EBV infection were attempts to find etiological factors, but in fact, other genes or factors might have been as likely as these if judged form the standpoint of today. A broader approach using methods like whole genome, array-based analyses for synchronous testing of multiple genes associated with carcinogenesis, or array-based comparative genomic hybridization to screen for genomic changes, were discussed. However, these ideas were not realized due to concerns about the value of these methods for our study, amongst other reasons because of poor DNA quality from the FFPE material and high costs on the other hand.

5 Conclusion

In conclusion, we found that in our area, patients with renal cell carcinoma have a remarkably higher risk to develop gastric cancer than the rest of the population. The analyzed patients had a comparable clinicopathological presentation, consisting of well differentiated clear cell renal cell carcinomas and poorly differentiated, aggressive gastric cancers. The presented cases could not be assigned to a known hereditary cancer syndrome, such as Lynch syndrome. In addition, *KRAS* mutations did not play a major role in our cases nor did EBV infection. Other genetic changes leading to a higher susceptibility for cancer may explain why some people have a higher risk to develop (multiple) malignancies and will have to be explored in future studies.

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7 Appendix

7.1 Betge et al. Oncol Rep. 2011 26:567-575

Gastric cancer and concomitant renal cancer: A systematic immunohistochemical and molecular analysis

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Abstract. The frequency of gastric cancer in patients with renal cell carcinoma (RCC) is exceptionally high in our region suggesting a common molecular basis. Our study aimed to characterize tumors and to analyze possible underlying molecular features in 12 patients with gastric cancer and concomitant RCC. We performed an immunohistochemical analysis including p53 protein expression, proliferative activity (MIB-1), mismatch repair status (hMLH1, hMSH2, hMSH6, PMS2) and E-cadherin expression in gastric cancers, which were additionally analyzed for Epstein-Barr-Encoded-RNA (EBER) by *in situ* hybridization. Microsatellite instability was analyzed with a PCR multiplex system and capillary electrophoresis. KRAS mutations in codons 12 and 13 were tested by pyrosequencing. All patients had clear cell RCCs, 10 of which were well differentiated and diagnosed in an early stage, while the gastric cancers of these patients were generally poorly or undifferentiated and diagnosed in an advanced stage. Gastric cancers showed reduced E-cadherin staining in 10 out of 12 cases. Two gastric cancers demonstrated loss of hMLH1 and PMS2, which was confirmed by molecular analysis showing a high degree of microsatellite instability. All RCCs were microsatellite stable. KRAS mutation was detected in one of the two instable gastric cancers, while none of the RCCs had KRAS mutations. Another gastric cancer was positive for EBV. In conclusion, a coherent cause for gastric cancer and concomitant RCC, such as Lynch syndrome, a prominent role of KRAS mutation or EBV infection, was not found in our series. Other factors leading to a higher susceptibility for cancer must be explored to explain why individuals with RCC have a higher risk of developing gastric cancer in our region.

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Key words: multiple primary neoplasms, hereditary neoplasms, *in situ* hybridization, microsatellite instability, *KRAS*, Epstein-Barr virus

Introduction

Patients with multiple malignancies constitute an attractive research topic. The analysis of different tumors occurring in a single patient may lead to the detection of hereditary causes for cancer or cancer syndromes and may improve understanding of general molecular pathologic principles of carcinogenesis.

Gastric cancer is the fourth most common cancer worldwide (1). Familial clustering is observed in approximately 10% of cases, but only 1-3% of these are hereditary (2). Cases with familial clustering are classified into familial diffuse gastric cancer (FDGC), including hereditary diffuse gastric cancer (HDGC), a cancer syndrome caused by a germline mutation in the E-cadherin (CDH1) gene (2,3), as well as familial intestinal gastric cancer (FIGC) or, if the histology is unknown, familial gastric cancer (FGC) (4). Moreover, gastric cancer may be observed as part of a hereditary tumor syndrome with the main localization other than the stomach. Thus, germline mutations of the BRCA1 and BRCA2 genes do not only increase the risk for breast and ovarian cancer, but do also increase the incidence of stomach, pancreas, prostate and colorectal cancer (5). Li-Fraumeni syndrome is caused by mutations of the tumor-suppressor gene *TP53*. Affected patients suffer from a wide spectrum of tumors, including breast and colorectal cancer, soft tissue or bone sarcoma, brain tumors, and, though infrequently, also gastric cancer (6). Finally, gastric cancer represents a well known extracolonic manifestation of the Lynch (HNPCC) syndrome (7). Neoplasms occurring as part of the Lynch syndrome are characterized by microsatellite instability (MSI) caused by germline mutations in mismatch repair genes (8).

None of the syndromes mentioned above have been reported to increase the risk for renal cell carcinoma (RCC). Nevertheless, the association of primary gastric and primary renal cancer has been presented in several case reports (9-22). Likewise, in the RCC database of our institute, we found 12 patients, who, in addition to RCC, also experienced gastric cancer, either synchronously or metachronously. The observed incidence was nearly twice as high as documented in the National Cancer Registry regarding the gastric cancer incidence in Austria (23).

Therefore, the present study aimed to evaluate patients with gastric and concomitant renal cancer. This is the first

Table I. Antibodies used for immunohistochemical staining.

Antibody	Source	Clone	Dilution/Epitope retrieval	Detection system	Chromogen
p53	Dako	DO-7	1:100/WB, Buffer pH 6.0	B	AEC
MIB-1	Ventana	K-2	Ready to use/Ventana iView Kit	A	DAB
E-cadherin	Zymed, San Francisco, CA, USA	4A2C7	Ready to use/MW, Buffer pH 9.0	C	AEC
hMLH1	Biocare, Concorde, CA, USA	G168-15	1:50/MW, Buffer pH 9.0	C	DAB
hMSH2	Ventana	G219-1129	1:50/Buffer CC1 standard	D	DAB
hMSH6	Biocare	BC-44	1:50/Buffer CC1 mild	D	DAB
PMS2	BD Biosciences, San Jose, Ca, USA	A 16-4	1:50/MW, Buffer pH 6.0	C	DAB

A, Ventana iView DAB; B, Dako REAL Detection System K5001; C, Dako EnVision+ (HRP rab/mouse) K5007; D, Ventana *ultra*View DAB 760-500. DAB, diaminobenzidine Dako (K5001); AEC, aminoethylcarbazole Dako (S2367); Buffer pH 9.0, Target Retrieval Solution Dako (S2367); Buffer pH 6.0, Epitope Retrieval Solution Dako (K5207). WB, water bath; MW, microwave; CC1, Ventana (950-124 SL).

study to systematically assess this association. First of all, we characterized the clinical and pathological presentation of these patients, analyzed epidemiologic and clinical data and presented histopathological and immunohistochemical features. In a second step, in order to analyze possible underlying genetic as well as infectious factors in the pathogenesis of both cancer types, we performed a thorough molecular analysis with respect to MSI, *KRAS* mutation and Epstein-Barr-Encoded-RNA (EBER) status.

Materials and methods

Patient selection. A systematic search of the RCC database of the Institute of Pathology, Medical University of Graz, Austria, covering 2082 patients (1180 males, 902 females; ratio 1.3:1) who underwent radical or partial nephrectomy between January 1984 and September 2005 was performed to identify those with synchronous and/or metachronous diagnosis of cancer in gastric biopsies or resection specimens. The medical records of these patients were reviewed in order to differentiate between patients with primary gastric cancer and those with secondary gastric involvement by RCC.

Patients with RCC metastatic to the stomach were presented in a previous publication (24), in which we described the clinical significance of this rare finding. During analysis, another 12 patients with synchronous and/or metachronous occurrence of both primary gastric and primary RCC were identified who represent the scope of the current analysis.

Clinicopathological and follow-up data of the 12 patients were analyzed in detail by chart review and interviewing attending physicians if possible. Basic personal data, such as patient age and gender were compared with data of patients suffering from either gastric or renal cancer contained in the computerized RCC and/or gastric cancer databases of our institution, respectively. The gastric cancer database covers 3072 patients (1689 males, 1378 females; ratio 1.2:1) who underwent total or partial gastrectomy between January 1984 and December 2008.

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Institutional Review Board approval was obtained from the Ethics Committee of the Medial University of Graz, Austria.

Histopathology. All tumor probes were carefully reassessed, paying special attention to tumor stage and grade as well as histological subtype of both renal and gastric cancers. Tumors were staged according to the 7th edition of the American Joint Committee on Cancer - Union Internationale Contre le Cancer (AJCC-UICC) Tumor-Node-Metastasis (TNM) Classification (25). Tumor grades were assessed following the WHO guidelines on renal (26) and gastric (27) cancers, respectively. Gastric cancers were additionally classified according to the Laurén classification (28).

Immunohistochemistry. Tissues were routinely fixed in 4% buffered formalin and embedded in paraffin. Sections (4-μm) were stained using automated staining systems (Dako TechMate™ 500; Dako, Glostrup, Denmark/Benchmark XT; Ventana Medical Systems, Tucson, AZ, USA) and commercially available antibodies (Table I). Epitope retrieval was performed and standardized for each antibody, using either microwave treatment, protease digestion or prediluted commercially available epitope retrieval solutions. Binding of the primary antibodies was visualized using the HRP/DAB+ Dako REAL™ detection system (catalog no. K5001, Dako) or the *ultra*View™ Universal DAB detection kit (catalog no. 760-500, Ventana), respectively.

Immunoreactivity was independently assessed by two investigators (M.J.P. and C.L.), and discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. Regarding p53 and MIB-1, positivity was identified as brown nuclear staining. The number of positive cells (labeling index) was determined by counting positive cells per 100 cancer cells, and immunoreactivity was semi-quantitatively assessed as follows: negative (<10%), weak or 1+ (11-20%), moderate or 2+ (21-50%) and high or 3+ (>50%). The amount of E-cadherin staining was evaluated in relation to staining of non-neoplastic mucosa and was assessed as reduced, when up to 50% of cancer cells lost specific membranous labeling, markedly reduced, when >50% lost specific membranous labeling, negative or positive. Regarding the four MMR proteins (hMLH1, hMSH2, hMSH6, PMS2), staining was recorded as either present (positive) or absent (negative).

Slides of a colorectal cancer known to exhibit high p53 as well as E-cadherin expression served as positive controls for

Table II. Patient and tumor characteristics.

	Renal cell carcinoma (RCC)						Gastric cancer					
Patient no.	Gender	Age (years)	pT/pN	Grade	Histological subtype	Size (cm)	Age (years)	pT/pN	Grade	Histology/Lauren classification	Location/ size (cm)	Outcome
1	M	64	1B/X	2	Clear cell	5.5	70	X/X	3	Signet ring cell with mucinous component/diffuse	Cardia/X	AWD (peritoneal metastases) 2 months after diagnosis of gastric cancer (no tumor resection)
2	M	57	1A/X	2	Clear cell	2	63	3/1	4	Undifferentiated/ diffuse	Antrum/ 5.5	DOD 10 months after diagnosis of gastric cancer
3	F	78	1A/X	2	Clear cell	3	78	3/1	3	Signet ring cell/diffuse	Corpus/ 2.2	Synchronous CRC (T3 N0) and renal oncocytoma, DOD 3 years after tumor diagnosis
4	M	68	1A/X	1	Clear cell	1.2	61	2/1	3	Signet ring cell with mucinous component/diffuse	Corpus/3	2 metachronous CRC, AWD (hepatic, pulmonal and bone metastases) 10 years after diagnosis of gastric cancer
5	F	66	1A/X	1	Clear cell	3	67	2/2	2	Tubular with mucinous component/intestinal	Cardia/6	BC 4 years prior to RCC, AWD (bone metastases) 8 years after diagnosis of BC
6	M	75	3A/X	2	Clear cell	7	75	3/2	3	Tubular/intestinal	Antrum/6	AWD (local recurrence of gastric cancer) 8 months after tumor diagnosis
7	M	69	1A/X	2	Clear cell	3.5	68	3/0	4	Tubular with undifferentiated component/mixed	Gastric stump/ 4.5	NED 10 years after diagnosis of gastric cancer
8	M	45	1A/X	2	Clear cell	4	55	X/X	4	Undifferentiated/ diffuse	Fundus/ X	Hepatic metastases at time of diagnosis of gastric cancer, no follow-up
9	M	75	1A/X	2	Clear cell	3.2	75	3/2	3	Signet ring cell/ diffuse	Corpus/6	DOC (myocardial infarction) 1 year after tumor diagnosis
10	M	41	1A/X	1	Clear cell	4	41	3/3	3	Signet ring cell with mucinous component/diffuse	Corpus/9	DOD 2 years after tumor diagnosis of gastric cancer
11	F	55	1A/X	1	Clear cell	3.5	62	3/3	3	Adenosquamous/ mixed	Antrum/ 7.6	AWD (hepatic, peritoneal and bone metastases) 4 years after diagnosis of gastric cancer
12	F	78	3A/X	3	Clear cell	4.5	78	1a/X	1	Papillary/intestinal	Antrum/ X	AWD (pulmonal, bone, hepatic and pancreas metastases) 1 year after tumor diagnosis

Age, age at diagnosis. NED, no evidence of disease; AWD, alive with disease; DOD, died of disease; DOC, died of other causes; CRC, colorectal cancer; BC, breast cancer.

p53, E-cadherin and MIB-1 immunostaining, respectively. Intratumoral lymphocytes served as positive controls for MMR proteins. Negative controls included omission of the primary antibodies and incubation with Dako REAL antibody diluent (no. S2022, Dako).

In situ hybridization. *In situ* hybridization was performed on paraffin-embedded sections (4-μm) using the INFORM EBER assay (Ventana) according to the manufacturer's instructions (regarding positive and negative controls and RNA preservation controls within samples) and an automated slide stainer system (Benchmark XT, Ventana). Briefly, after deparaffinization and rehydration, tissues were treated with proteinase K and then hybridized with the fluorescein-labeled EBER oligonucleotide probe (INFORM EBER Probe, Ventana). The hybridized probe was visualized using an *in situ* hybridization detection system (ISH iVIEW Blue detection kit, Ventana), which utilizes an anti-fluorescein primary antibody binding to the EBER probe and a biotinylated secondary antibody formulation binding to the primary mouse antibody. Streptavidin-conjugated alkaline phosphatase was then used as a chromogenic enzyme, which generates a blue nuclear signal, which is evaluated by light microscopy. A nasopharyngeal carcinoma was used as a positive control.

Molecular analysis. DNA from formalin-fixed paraffin-embedded tissue was extracted using the Qiagen QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, in an elution volume of 50 μl.

MSI was investigated using the Promega Microsatellite Analysis System version 1.2 (Promega, Mannheim, Germany), a PCR multiplex system using 5 mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, MONO-27) to determine MSI and 2 pentanucleotide repeat markers (Penta C and Penta D) for internal control. Two separate analyses using 50 and 100 ng of DNA, respectively, were performed. PCR products were separated by capillary electrophoresis using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Vienna, Austria). According to the instructions, MSI at 2 mononucleotide loci was reported as MSI-high, instability at one locus as MSI-low and no instability at any of the loci tested as microsatellite stable (MSS).

We tested for *KRAS* mutations in codons 12 and 13 by pyrosequencing using the Pyromark Q24 *KRAS* kit v2.0 (Qiagen). Two analyses, using 20 and 40 ng of DNA, respectively, were performed. The amplification was carried out in a 25-μl reaction volume, containing 5 μl of DNA solution, 0.5 μl of codon 12+13 biotinylated reverse primers, 0.5 μl forward primer, 2.5 μl Qiagen buffer, 0.2 μl of each dNTP, and 0.16 μl Qiagen Taq polymerase. The amplification profile was 15 min at 95˚C; 45 cycles with 20 sec at 95˚C, 30 sec at 53˚C and 20 sec at 72˚C; followed by 5 min at 72˚C. PCR products (8 μl) were analyzed by electrophoresis in a 3% agarose gel to confirm successful amplification. To prepare single-stranded DNA, the PCR products were immobilized with streptavidin sepharose beads and purified with the PyroMark Q24 Vacuum Workstation. Pyrosequencing primer (25 μl) was annealed to the purified single-stranded PCR product. After pyrosequencing using the nucleotide dispensation order TACGACTCAGATCGTAG results were analyzed with PyroMark Q24 software.

Results

Clinical data/outcome analysis. The incidence of gastric cancer in RCC patients included in the RCC database of our institution was 27/100,000, compared with the gastric cancer incidence of 14/100,000 recorded in the Austrian cancer registry for the same study period (23).

Of the 12 patients, 8 were male and 4 were female. Gastric cancer was diagnosed at a mean age of 66 years (median 65, range 41-78), compared with 68 years (median 69, range 11-97) in the gastric cancer database. Accordingly, RCC was diagnosed at a mean age of 64 years (median 67, 41-78), compared with 62 years (median 63, range 9-88) in the RCC database. In 5 patients, RCC was diagnosed prior to gastric cancer; in 5 patients, synchronous to gastric cancer, and in 2 patients RCC was diagnosed after gastric cancer, respectively.

Detailed information regarding the patients, such as outcome analysis and presence of tertiary malignancies, is presented in Table II.

Histology. Details concerning the histology of all tumors including T and N classification, tumor size and location, as well as Laurén's classification for gastric cancer are presented in Table II. Briefly, all RCCs were of clear cell type. In general, they were well differentiated and were predominantly diagnosed at an early tumor stage. None of the RCCs had lymph node metastases.

Gastric tumors were poorly differentiated in the vast majority of cases and were generally diagnosed at a higher tumor stage. Five tumors were diagnosed as signet ring cell carcinomas (patients nos. 1, 3, 4, 9 and 10) of which three showed extracellular mucin production (nos. 1, 4 and 10; Fig. 1A). One patient (no. 5) had a moderately differentiated tubular adenocarcinoma with a mucinous component, one patient (no. 11) an adenosquamous (Fig. 1B) and another patient (no. 12) a well-differentiated papillary carcinoma. Three tumors were diagnosed as undifferentiated carcinomas (nos. 2, 7 and 8) of which one showed areas of a pre-existing moderately differentiated tubular adenocarcinoma (no. 7). The undifferentiated carcinoma of patient no. 2 showed large areas of necrosis and was found to be densely infiltrated by mixed inflammatory cells (Fig. 1C). The undifferentiated carcinoma with pre-existing better differentiated areas of patient no. 7 was characterized by a dense intratumoral and peritumoral lymphoplasmacellular infiltrate including lymph follicle formation (Fig. 1D).

The colorectal cancer of patient no. 3 was a poorly differentiated adenosquamous carcinoma with a mucinous component (<50% of the tumor area), while both colorectal cancers of patient no. 4 were poorly differentiated adenocarcinomas with a mucinous component (<50% of the tumor area).

Immunohistochemistry. Regarding gastric tumors, high (3+) proliferative activity (MIB-1) was present in the majority of cases (Fig. 2A). P53 protein overexpression was observed in 7 carcinomas and varying loss and/or reduction of membranous E-cadherin immunolabeling in 10 carcinomas (Fig. 2B and C). Two gastric cancers (patients nos. 2 and 11) showed loss of nuclear expression of mismatch repair proteins hMLH1 and PMS2 (Fig. 2D and E). This finding was confirmed by molecular analysis showing a high degree of MSI (Table III, compare below). All RCCs as well as the three colorectal cancers from patients nos. 3 and 4 retained mismatch repair protein expression.

In situ hybridization. Presence of EBV was demonstrated in the gastric cancer characterized by dense intratumoral lymphoplasmacellular infiltrate in H&E-stained sections (patient no. 7), whereas all other tumors were negative (Fig. 2F).

Molecular analysis. Testing for MSI, we found additional peaks for the microsatellite markers NR-21, BAT-26, BAT-25, NR-24 and MONO-27 in the gastric cancer of patient no. 2, compared with the patient's normal and RCC tissue (Fig. 3). In patient no. 11 we observed a characteristic peak broadening regarding the markers NR-21, BAT-26, BAT-25 and MONO-27 in gastric cancer tissue, compared with the patient's normal and RCC tissue. Hence, we classified both patients MSI-high according to the Bethesda guidelines, which is concordant with the immunohistochemical findings in these patients. We did not find MSI in any of the RCCs. The colorectal cancers of patients no. 3 and 4 were also MSS.

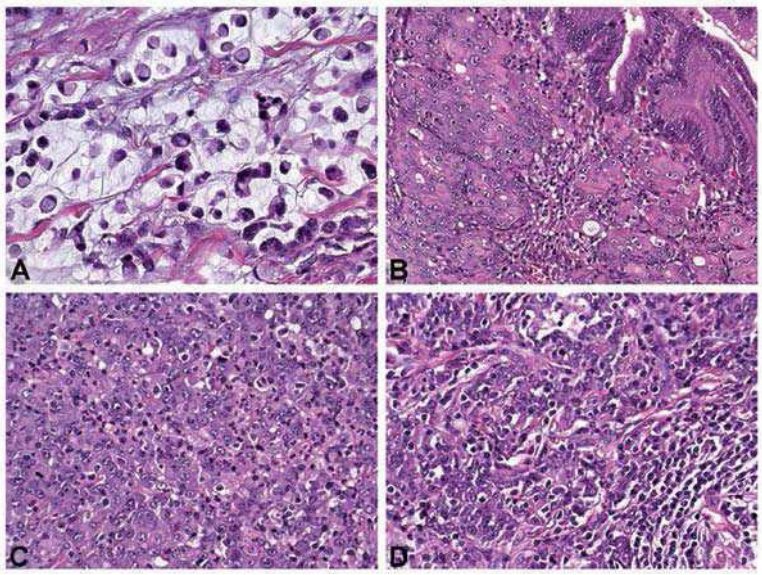

Figure 1. Morphologic diversity of gastric cancer associated with renal cell carcinoma. (A) Signet ring cell carcinoma with extracellular mucin production (x100), (B) poorly differentiated adenosquamous carcinoma (x100) and (C) undifferentiated carcinoma with marked intratumoral inflammation with mixed infiltrate (x100) or (C) predominantly lymphoplasmacellular infiltrate (x100).

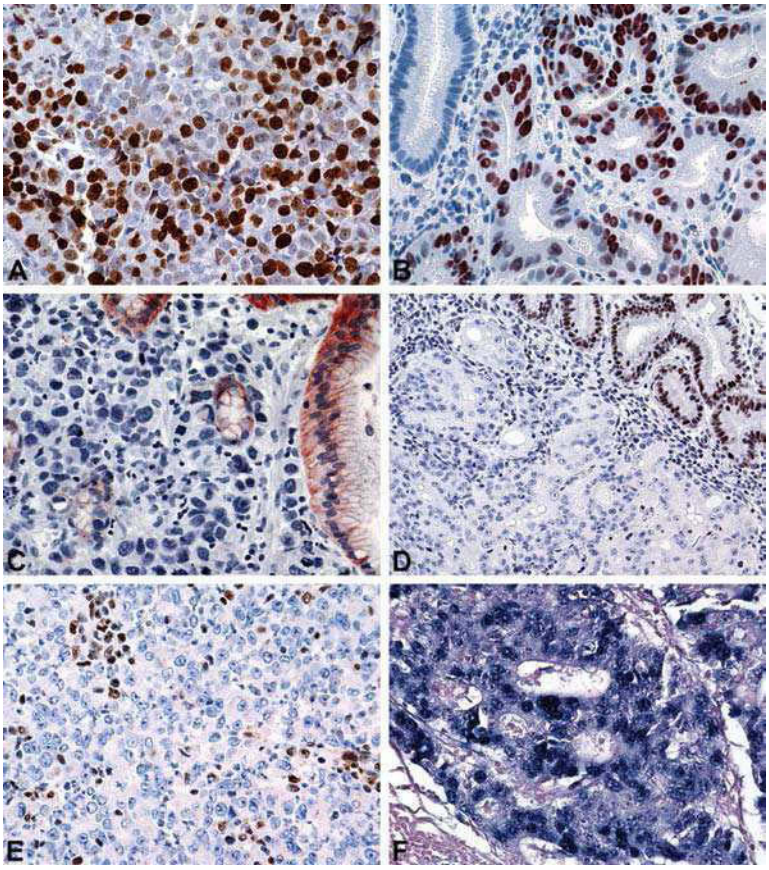

Figure 2. Immunohistochemistry and *in situ* hybridization of gastric cancer associated with renal cell carcinoma. (A) High proliferative activity of MIB-1 (x100) and (B) p53 overexpression of cancer cells (x100). (C) Loss of E-cadherin staining in poorly differentiated gastric cancer (x100). (D) Loss of hMLH1 (x100) and (E) PMS2 immunostaining in undifferentiated tumor tissue (x100). (F) EBER (x100).

Screening for *KRAS* mutations in codons 12 and 13 was positive in one of the gastric cancers; we found a transversion form guanine to cytosine at the second base of codon 12 resulting in Gly12Ala in the gastric cancer of patient no. 2. Additionally, we found *KRAS* mutations at the second base of codon 12 in both of the colorectal cancers; patient no. 3 had a transversion to thymine (Gly12Val) and patient no. 4 a transition to adenine (Gly12Asp). We did not detect *KRAS* mutations

Table III. Immunohistochemical and molecular findings in gastric cancers.

	Immunohistochemistry							Molecular analysis ISH		
Patient no.	p53	MIB1	E-cad	MLH1	MSH2	MSH6	PMS2	MSI	*KRAS*	EBER
1	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
2	-	+++	pos	neg	pos	pos	neg	MSI-H	mut	neg
3	-	+	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
4	-	+++	↓↓	pos	pos	pos	pos	MSS	wt	neg
5	++	+	↓	pos	pos	pos	pos	MSS	wt	neg
6	-	++	↓	pos	pos	pos	pos	MSS	wt	neg
7	+++	+++	↓	pos	pos	pos	pos	MSS	wt	pos
8	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	u	neg
9	+++	+	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
10	+++	+++	↓↓↓	pos	pos	pos	pos	MSS	wt	neg
11	-	+	↓	neg	pos	pos	neg	MSI-H	wt	neg
12	+++	+++	pos	pos	pos	pos	pos	MSS	wt	neg

ISH, *in situ* hybridisation; E-cad, E-cadherin; pos, positive; neg, negative (or ↓↓↓ for E-cadherin); +, weak; ++, moderate; +++, extensive; MSS, microsatellite stable; MSI-H, microsatellite instability-high; wt, wild-type; mut, mutated; u, unknown.

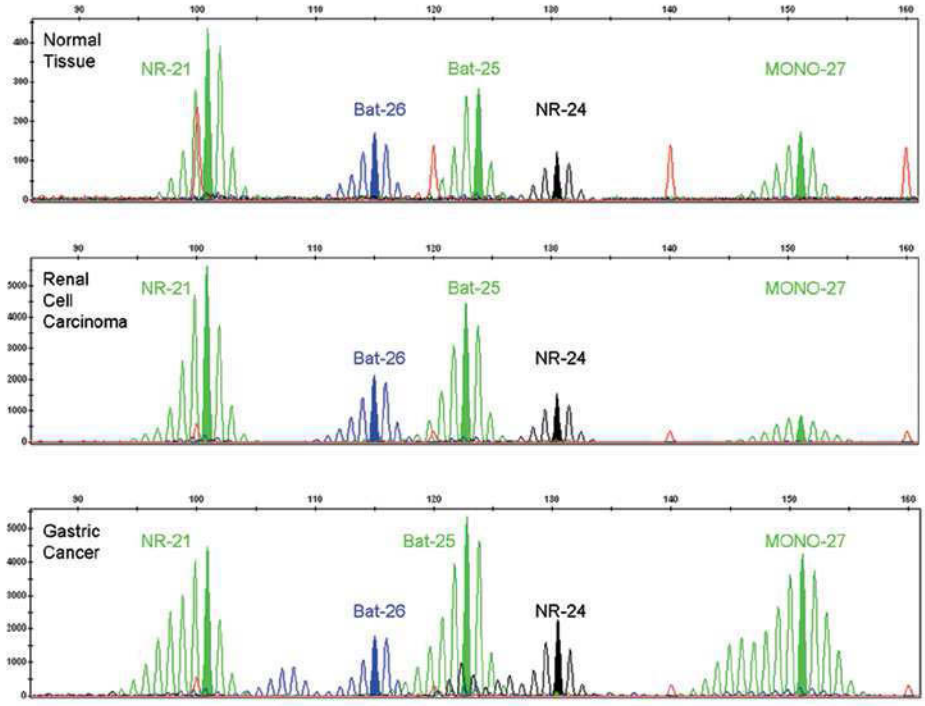

Figure 3. MSI testing shows additional peaks for the microsatellite markers NR-21, BAT-26, BAT-25, NR-24 and MONO-27 in the gastric cancer of patient no. 2, compared to the patient's normal and renal cancer tissue.

in any of the RCCs. The material of one gastric cancer could not be analyzed due to poor DNA quality. One RCC could not be subjected to molecular analysis due to insufficient material.

Discussion

Carcinogenesis is driven by progressive accumulation of somatic mutations in a number of tumor-related genes. Patients with multiple neoplasms are more likely to harbor a germline mutation in one of those genes. It is estimated that approximately 5% of all cancers arise within a hereditary cancer syndrome, each of which present with a spectrum of tumors (29).

A systematic study analyzing the association of gastric and renal cancer is lacking. In the present study, we focused on 12 patients with gastric and synchronous and/or metachronous RCC based on the observation of a markedly high incidence of gastric cancer in patients with RCC, suggesting

the possibility of a hitherto unrecognized genetic association or hereditary cancer syndrome.

The manifestation age regarding both gastric and renal cancer, however, was not significantly different from that of patients included in the gastric and renal cancer databases, respectively. Moreover, the male predominance of our cohort is in the line with epidemiologic data from our country, where the risk for male patients to develop gastric or renal cancer is almost twice as high as for females (23).

All RCCs were of clear cell type, which is known to represent the most common form of renal cancer worldwide. Nevertheless, it was noteworthy that 11 of the 12 tumors were well differentiated (grade 1 or 2), while the majority of gastric cancers were very poorly differentiated (grade 3) or even undifferentiated (grade 4) at the time of diagnosis, apart from patient no. 12, who had a well-differentiated gastric cancer and a grade 3 RCC.

Some of the poorly differentiated gastric tumors harbored marked intratumoral inflammation or presented with rather uncommon histological subtypes, such as adenosquamous carcinoma. According to literature data, intratumoral mixed or predominantly lymphoplasmacellular infiltration has been related to MSI or EBV infection (30-33), respectively. This led to the hypothesis, that Lynch syndrome may have caused gastric cancer associated with renal cancer in our cohort. In addition, we searched for EBV in cancer tissues, particularly since EBV is associated with hypermethylation of tumor-suppressor genes known to be involved gastric cancer (34).

To note, gastric cancer is the second most common extra-colonic malignancy in patients with Lynch syndrome. Because of the relatively high incidence of gastric cancer in the general population, the true association of gastric cancer and Lynch syndrome is, however, controversial (35). Nevertheless, approximately 15-20% of gastric cancers are MSI. In these cases, hypermethylation of the hMLH1 promotor region appears to be the responsible mechanism (2,36).

We found the gastric cancer with marked mixed infiltrate to be MSI (patient no. 2) and the gastric cancer with predominantly lymphoplasmacellular infiltrate to be associated with EBV infection (patient no. 7). The second gastric cancer with MSI showed adenosquamous differentiation (patient no. 11) which has been related to MSI-high status in 12% (2 out of 17) of cases (37). However, the overall incidence of MSI-high status (2 out of 12; 17%) in our cohort of gastric cancers is in line with literature data ranging from 10 to 20% of tumors (38-42). All RCCs analyzed in our study were MSS. Data on the presence of MSI or mismatch repair gene defects in RCCs are conflicting. Reported incidences range from 0 to 40% (43-51). The fact that, in our series, both renal tumors from patients with MSI-high gastric cancers were MSS argues against germline mutations in DNA repair genes (Lynch syndrome) and we conclude that neither Lynch syndrome nor EBV infection represents a major cause with respect to carcinogenesis in our patient cohort.

A crucial pathway involved in cell proliferation is the *RAS-RAF-MEK-ERK-MAP* kinase pathway which is frequently activated in cancer. In contrast to colorectal cancer, *BRAF* mutations are exceedingly rare in gastric cancers and do not characterize MSI tumors (52). The frequency of *KRAS* mutations in gastric cancer ranges between 0 and 8.5% (36,37,53-57). In RCCs the incidence of *KRAS* mutations is lower (58), while activating mutations in the *KRAS* gene are found in 30-40% of colorectal carcinomas (59). Patient no. 2 had a gastric cancer harboring a *KRAS* mutation and was also MSI. Additionally, we observed *KRAS* mutations in both of the analyzed colorectal carcinomas, but in none of the RCCs. Hence, the observed frequencies in our patients are in agreement with the literature. In addition, data suggest that *KRAS* mutations are more frequent in MSI-high gastric cancers than in MSS. Thus, Brennetot *et al* (60) observed *KRAS* mutations in 28% of MSI gastric cancer, while no mutations were noted in MSS tumors.

Finally, there are some hereditary cancer syndromes that may cause clear cell RCC, but none of these is related to a higher frequency of gastric cancer. Most common is von Hippel-Lindau disease, which leads to (often multifocal) clear cell RCC and renal cysts and is additionally associated with CNS and retinal haemangioblastomas, pheochromocytomas, pancreatic cysts and neuroendocrine tumors as well as endolymphatic sac tumors of the inner ear, epididymal and broad ligament cystadenomas (61). The Birt-Hogg-Dubé syndrome also includes clear cell RCC or oncocytoma, but most often chromophobe or oncocytic hybrid tumors as renal manifestation, while fibrofolliculomas, trichodiscomas and acrochordons may be noted as extra-renal manifestations. Moreover, spontaneous pneumothoraces due to lung cysts are frequent in these patients (61). Finally, clear cell RCC may also occur in patients with constitutional chromosome 3 translocation, a very rare syndrome leading to multiple bilateral clear cell RCCs without additional systemic manifestations (62). All these syndromes, however, do not fit with the clinicopathological presentation of our patients.

In conclusion, we found that in our region, patients with RCC have a markedly higher risk to develop gastric cancer than the rest of the population. The 12 analyzed patients had a comparable clinicopathological presentation, consisting of well-differentiated clear cell RCCs and poorly differentiated, aggressive gastric cancers. The presented cases could not be assigned to a known hereditary cancer syndrome, such as Lynch syndrome. In addition, *KRAS* mutations did not play a major role in our cases nor did EBV infection. Other genetic changes leading to a higher susceptibility for cancer may explain why some people have a higher risk to develop (multiple) malignancies. This will have to be explored in future studies.

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