Original Paper

Pathobiology

Mismatch Repair Proteins hMLH1 and hMSH2 Are Differently Expressed in the Three Main Subtypes of Sporadic Renal Cell Carcinoma

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Abstract
Objectives: We studied the role of minor mismatch repair proteins (MMR) human MutL homologue 1 (hMLH1) and human MutS homologue 2 (hMSH2) in the main subtypes of renal cell carcinoma (RCC). *Methods:* Expression of MMR proteins hMLH1 and hMSH2 were investigated in 166 RCC tumors, containing the main subtypes by immunohistochemistry. Furthermore, each tumor was screened for microsatellite instability (MSI) using the National Cancer Institute consensus panel for hereditary non-polyposis colon carcinoma as well as for elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) by 10 additional markers. *Results:* MSI was found only in 2.0% of analyzable cases and EMAST was detected only in 1 patient. hMLH1 and hMSH2 expression was reduced in 83.7 (118/141) and 51.2% (65/127) of cases, respectively, in a subtype-specific manner. None of the clear cell RCC tumors retained a high hMLH1 expression and 92.0% lost hMLH1 completely, while papillary and chromophobe RCC preserved the expression in 25.0 and 33.3% of cases (p < 0.001). Subtype specificity was also present in hMSH2 staining, where chromophobe RCC retained a high expression in 41.7% of cases, while clear cell and papillary tumors did not (29.9 and 23.1%; p = 0.01). *Conclusion:* MSI and EMAST are rare events in sporadic RCC, whereas diminished MMR protein expression is linked to tumor entity and might contribute to the different biological behavior of the RCC subtypes.

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Introduction

Renal cell cancer (RCC) is a genetically heterogeneous disease. Molecular biological studies have shown multiple genetic and epigenetic alterations, i.e. subtype-specific gains and losses of chromosomes, chromosome arms and chromosomal regions [1]. Besides chromosomal instability involving alterations in chromosomal segregation and the formation of cells containing fragmentized, deleted or duplicated chromosomes, microsatellite instability (MSI) has been described as a distinct type of genetic instability [2, 3]. Microsatellites are mono- to pentanucleotide repeats found throughout the genome. Usually they are not located at exons or promoter re-

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Table 1. Characteristics of MSI and EMAST-positive tumors

Case No.	Diagnosis	MSI	Marker with MSI	EMAST	Marker with EMAST	TP53 alu	hMLH1	hMSH2
22	sarcomatoid RCC pT3a N0 M0 G3	MSI-L	MFD15/APC	–	–	n.i.	negative	moderate
77	papillary RCC pT3a N2 M0 G2	MSI-H	BAT40 D2S123 MFD15/APC	EMAST	D9S747 PKY11 D21S1436	–	negative	negative
138	chromophobe sarcomatoid RCC pT3b N2 M0 G3	MSI-L	BAT40	–	–	n.i.	negative	negative

MSI-L = MSI-low; n.i. = not informative.

gions. This finding was attributed to their susceptibility to transcription errors caused by polymerases which might lead to shifts in open reading frames [4]. Their function remains largely unknown, but a possible role in evolution, especially when present at coding sequences of minor mismatch repair (MMR) proteins, has been discussed [5]. MMR proteins are concerned with correcting replication errors by means of homologous recombination [6, 7]. Error rates are remarkably increased at mono- and dinucleotide repeats, as they cause polymerases to slip above average [8]. Hereditary non-polyposis colon carcinoma (HNPCC)-associated cancers are well known to be caused by mutations in MMR genes – e.g. human MutL homologue 1 (hMLH1), human MutS homologue 2 (hMSH2), human MutS homologue 6 (hMSH6) – leading to MMR deficiency and impairment of transcription error repair [2]. Meanwhile, a marker panel targeting especially vulnerable mono- and dinucleotide sequences has been defined and is generally used for the detection of MSI [9]. The fact that HNPCC patients occasionally develop RCC [10] incited several examinations of MSI and MMR deficiency in RCC tumors and cell lines [11–22], with contradictory results. Studies were based on MSI and immunohistochemistry analyses as well as on characterization of the hypermethylation status of hMLH1 and hMSH2 including DNA sequencing of the complete coding region of the two genes, but only one recent investigation used the National Cancer Institute (NCI) consensus panel [21]. Moreover, there are no studies dealing with MSI status and MMR protein expression in the same specimens [11–19], and merely one study on cell lines exists [20]. Recently, elevated microsatellite alterations at selected tetranucleotides (EMAST) have been defined as another type of molecular alterations in several tumor types [23–25]. Furthermore, it has been shown to be a distinct form of MSI in tumors of the urinary tract [26, 27]. EMAST is not linked to known MMR gene defects [23]; however, p53-dependent DNA repair mechanisms have been described to be impaired in tumors with EMAST [24]. To date, there is no study investigating EMAST and p53 status together in a well-defined cohort of RCC patients.

Our study is designed to clarify the incidence of MMR deficiency and MSI as defined by the NCI consensus panel and exploring the role of EMAST in view of the three main subtypes of RCC.

Materials and Methods

The ethics committee of the University Hospital of Erlangen approved the study protocol.

One hundred and sixty-six tissue samples from RCC patients obtained by tumor nephrectomy were retrieved from the archives of the Institute of Pathology, University of Regensburg, Regensburg, Germany. All tumors were diagnosed according to the 2004 WHO classification of RCC [28] and staged according to the tumor-node-metastasis (TNM) system [29, 30]. Prior institutional review board approval was obtained through the participating institutions. RCC specimens consisted of 104 clear cell, 33 papillary, 32 chromophobe, 2 spindle cell, 4 undifferentiated, 3 mixed clear cell and papillary RCC, 4 oncocytomas and 1 collecting duct carcinoma. TNM classifications ranged from T1a to T3b, cN0 to pN2, M0 to M1 and tumor stages from I to III (details are given in table 1). The mean age of patients was 63.6 years (range 26–91, median 76).

Microdissection and DNA Isolation
DNA was extracted as described previously [31] from manually microdissected, formalin-fixed, paraffin-embedded normal and tumor tissues by means of the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and according to the manufacturer's instructions.
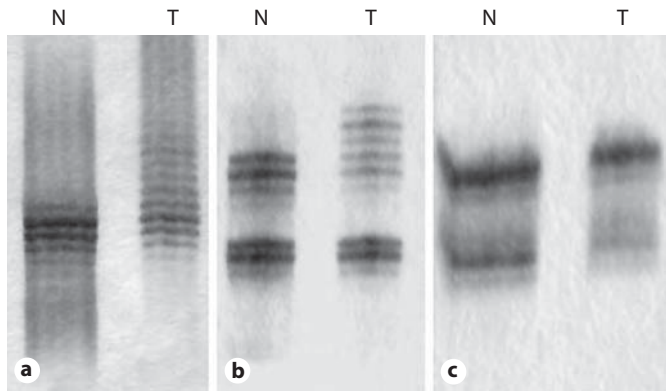
Fig. 1. Representative examples for detected chromosomal aberrations in RCC. **a** MSI in marker D2S123. **b** MSI in EMAST marker PKY11. **c** LOH in EMAST marker D21S1436. N = Normal tissue; T = tumor.

Microsatellite Analysis

Microsatellite analysis was performed on DNA of tumor and corresponding normal tissue. We used the NCI consensus panel, supplemented by BAT40, to detect mono- and dinucleotide MSI [9]. For assessment of EMAST, we chose eight microsatellite markers (Mycl1, D2S443, UT5037, D8S321, D8S348, D9S303, D20S82 and D21S1436) from the literature [21, 23–25]. Additionally, two tetranucleotide markers on chromosome 9 (D9S304, D9S747) were investigated to evaluate the frequency of loss of heterozygosity (LOH) on chomosome 9 as they showed high sensitivity in detecting EMAST in a recent bladder cancer study [26]. For investigation of the deletion status of the p53 gene locus on chromosome 17p13.1, the pentanucleotide marker TP53alu was used [32]. All primer sequences were taken from the Genome Database (http://www.gdb.org). DNA amplification conditions and the following procedures for separation and visualization of products were published previously [33]. In short, polyacrylamid gel electrophoresis and silver staining were used. Every gel was assessed independently by two investigators (C.S. and R.S.). Informative cases were scored as allelic loss (LOH) when the intensity of the signal for one allele in the tumor was decreased to at least 50% relative to the allele of the control. MSI was defined as the appearance of additional bands or band shifts in the tumor. Tumors were classified as MSI-high (MSI-H) if two of the six markers (>30%) were found to be unstable. If <30% of the investigated markers revealed MSI, the tumor was designated as having low-level instability (MSI-low). LOH was not counted as MSI, but considered as a separate group. All analyses were run in duplicate after independent polymerase chain reactions to avoid errors produced by preferential amplification of one allele. Cases with non-reproducible amplification patterns were excluded from the study due to minor DNA quality or low DNA quantity.

Immunohistochemistry

For immunohistochemical analyses, a RCC tumor microarray was built of representative areas from formalin-fixed, paraffin-embedded tumor material as described previously [34]. The freshly cut 5-µm sections of the five resulting blocks underwent strict-

ly synchronous treatment throughout all preparatory steps and staining procedures. The latter were carried out on a NexES immunohistochemistry system (Ventana Medical Systems, Illkirch, France), using the iView DAB Detection Kit (Ventana Medical Systems). Protein expression of hMLH1 and hMSH2 was evaluated according to immunohistochemistry standard procedures with primary monoclonal antibodies anti-MLH1 (13291A, clone G168-728, Pharmingen, San Diego, Calif., USA; dilution 1:20) and anti-hMSH2 (clone 2MSH01, Neomarkers, Fremont, Calif., USA; dilution 1:100) and the help of an Amplification Kit (Ventana Medical Systems). As a control for MMR protein staining, we used colorectal normal tissue, which is known to express MMR proteins. Scoring of immunoreactivity was performed in a way similar to that of a previously published study [35]: negative, no positive nuclei; weak, ≤20% of nuclei stained positively; moderate, ≤50% of nuclei stained positively; strong, >50% of nuclei stained positively. For analysis of combined hMLH1 and hMSH2 loss, samples of negative and weak expression of the respective protein were banded together to a group named 'loss of expression' and moderate and strong expression samples to a group termed 'normal expression'.

Statistical Analysis

Two-sided Fisher's exact test was used to study the statistical association between MSI and immunoreactivity for hMLH1, hMSH2 and combined MMR loss. Staining and microsatellite analyses were also studied regarding possible associations with histopathological characteristics. p values <0.05 were defined significant. As the study was of explorative character, no correction for multiple testing was applied [36]. All statistical procedures were performed using Statistical Package for the Social Sciences (SPSS) 13.0.

Results

Microsatellite analysis using markers of the NCI consensus panel resulted in stable mono- and dinucleotide sequences in the majority of all types of renal tumors. Merely 2 cases of MSI-low (1.3%) and 1 of MSI-H (0.7%) were found in 152 informative cases, respectively, and were of International Union against Cancer (UICC) stages III or IV (p = 0.014). Representative examples for MSI in these tumors are given in figure 1. Testing all tumors with regard to the expression of hMLH1 and hMSH2 by immunohistochemistry, we found a proportion of tumors displaying reduced expression of one protein and also some tumors showing combined loss in a subtype-specific manner (fig. 2). None of the clear cell RCC tumors retained high hMLH1 expression and 92.0% (80/87) lost hMLH1 completely. Papillary and chromophobe RCC preserved full protein expression in 25.0 (7/28) and 33.3% (4/12), while complete loss was displayed in 64.3 (18/28) and 58.3% (7/12), respectively (p < 0.001). Seventy-five percent of oncocytomas (3/4) also presented hMLH1
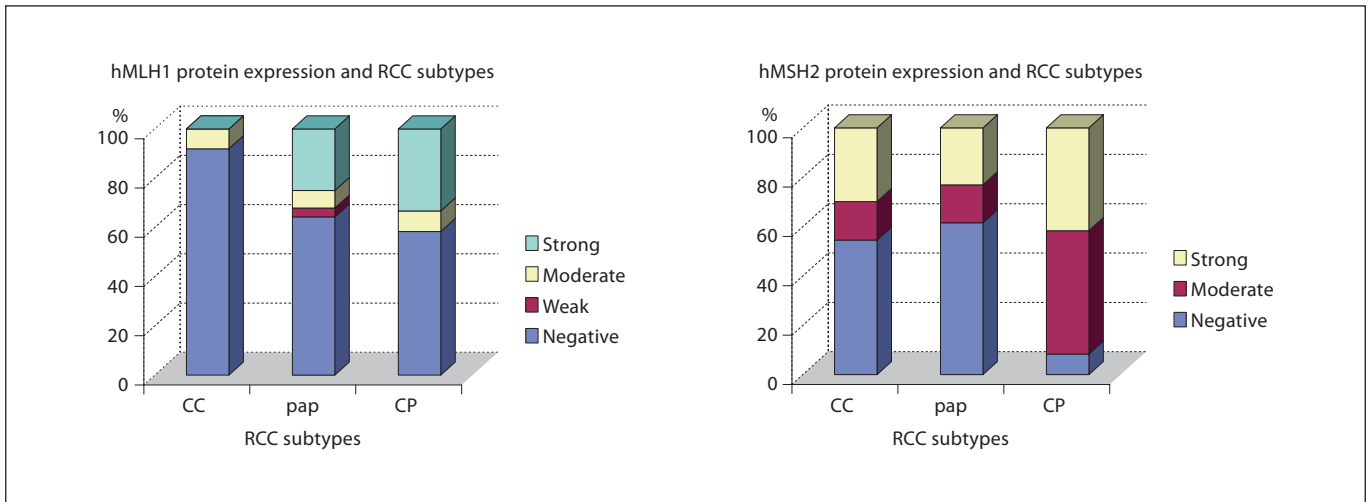
Fig. 2. Loss of hMLH1 and hMSH2 protein expression is subtype specific (p < 0.001). CC = Clear cell RCC; pap = papillary RCC; CP = chromophobe RCC. hMLH1: clear cell RCC, n = 87; papillary RCC, n = 28; chromophobe RCC, n = 12. hMSH2: clear cell RCC, n = 77; papillary RCC, n = 26; chromophobe RCC, n = 12.

protein loss. Subtype specificity was also present in hMSH2 stainings, where chromophobe RCC retained full protein expression in 41.7% (5/12), while clear cell and papillary tumors showed high expression only in 29.9 (23/77) and 23.1% (6/26) of cases, respectively, and complete loss was visible in only 8.3% (1/12) chromophobe, 54.5% (42/77) clear cell and 61.5% (16/26) papillary cases (p = 0.01). Oncocytomas lost hMSH2 expression in 75% (3/4) of cases. For hMSH2, there was also an association with pT (p = 0.026) and young age at diagnosis (<45 years of age; p = 0.029). No further significant associations were found. Representative examples for hMLH1 and hMSH2 deficiency are given in figure 3.

The single MSI-H tumor displayed combined loss of both proteins. Although combined loss of hMLH1 and hMSH2 was detected in 50.0% (63/126) of cases, no significant correlation with MSI status was found. There was also no association with histopathological characteristics.

Additionally, we applied EMAST markers to examine the frequency of instability at tetranucleotide sites. EMAST was present in only 1 of 130 informative cases (0.8%; fig. 1). Remarkably, this tumor was of UICC stage IV (pT3a N2 M0), demonstrated a loss of hMLH1 and hMSH2 protein and was MSI-H. The association with N2 was significant (n = 119; p = 0.008). We also performed TP53alu LOH analysis: 3.1% (3/97) of tumors lost an allele at this locus. The single EMAST-positive case did not demonstrate LOH at TP53alu. Detailed charac-
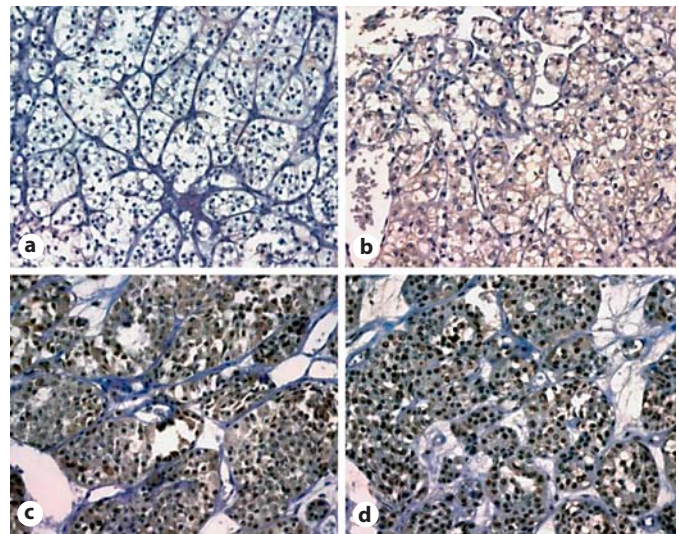

Fig. 3. Immunohistochemistry of MMR proteins. **a** Strong nuclear staining in a hMLH1-positive clear cell RCC. **b** Absence of staining in a hMLH1 chromophobe RCC. **c** Strong nuclear staining in a hMSH2 clear cell RCC. **d** No immunohistochemical staining in a hMSH2.

teristics of MSI and EMAST-positive cases are reviewed in table 1.

Analysis also revealed numerous cases with LOH (19.8%, 32/162) at the applied markers. Some of them were located at chromosomal regions known to get fre-

quently lost in a subtype-specific manner in RCC, but also additional ones were observed: clear cell RCC, that are known to display loss of different regions of 3p, 6q, 8p, 9p and 14q, demonstrated LOH at UT5037 (chromosome 8; 1.7%, 1/59), but also at Mycl1 (chromosome 1p34.1–34.3; 2.6%, 2/78), D8S348 (chromosome 8q24.13–24.3; 5.4%, 4/74), D8S321 (chromosome 8q24.13–8; 5.75, 4/70), D9S747 (chromosome 9q32; 3.2%, 2/63), D9S303 (chromosome 9q21.32; 1.3%, 1/75) and D20S82 (chromosome 20p12.3; 1.0%, 1/96). Papillary tumors, that are noted for gaining chromosomes displayed LOH at Mycl1 (8.3%, 2/24), D2S443 (chromosome 2p 13.2–13.1; 5.0%, 1/20), UT5037 (17.6%, 3/17), D9S747 (5.6%, 1/18), D9S304 (chromosome 9p21; 4.0%, 1/25) and D9S303 (14.3%, 3/21). Chromophobe tumors often harboring combined losses of chromosomes 1, 2, 6, 10, 13, 17 and 21 were found to display LOH at Mycl1 (71.4%, 5/7) and D2S443 (14.3%, 1/7), but also at D8S348 (20.0%, 2/10) and D9S303 (10.0%, 1/10). Oncocytomas which are known for loss of 1p, 14q and Y displayed LOH at D9S303 (1/4) and Mycl (2/4).

Discussion

RCC has occasionally been reported to occur in patients with HNPCC and some RCCs have been shown to have MSI at multiple loci. Therefore, separate studies focused on MSI and MMR deficiency in RCC. Leach and colleagues [20] reported three RCC lines with hMLH1 deficiency, two of which carried hMLH1 mutations and were genetically unstable. They also examined the expression of hMSH2, hMSH6 and hPMS2; all of them were detectable in their 25 cell lines. Deguchi et al. [12] analyzed hMLH1, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2 mRNA as well as protein expression in both RCC tissues and cell lines and found mRNA loss of hMLH1 and hMSH3, which did not rise after cell line treatment with 5-aza-2′deoxycytidine, suggesting that mechanisms other than promoter hypermethylation have to be involved. Similarly, Rubio-del-Campo and coworkers [21] focused on MSI and promoter hypermethylation status of the hMLH1 and hMSH2 encoding genes, using the Bethesda microsatellite panel for the first time. As a result, they found loss of function of both hMLH1and hMSH2 not to be involved in sporadic RCC, neither by promoter methylation nor by mutation in their exons. Interestingly, LOH was common among their patients with RCC, leading to the conclusion that genomic instability in RCC does not affect small fragments of the genome

(MSI), but is related to the level of large fragments (chromosomes). Loss of 3p for example, or part of it, is a characteristic feature of clear cell RCC [28] and hMLH1 is known to be located on 3p21–23 [37], which elucidates the phenomenon of hMLH1 deficiency in spite of stable nucleotide microsatellites. Loss of hMLH1 and hMSH2 protein expression regarding the different RCC subtypes was observed first by Baiyee and Banner [38]; in detail, deficiency in hMLH1 was found in 40% for clear cell (n = 20) and in 50% for papillary tumors (n = 8), while hMSH2 was lost in 20% of their clear cell and none of their papillary RCC specimens. Chromophobe RCCs (n = 4) were found to be deficient in hMSH2 but not in hMLH1. In contrast, our study – containing 104 clear cell, 33 papillary and 32 chromophobe RCC specimens – noticed loss of hMLH1 and hMSH2 much more frequently in all three RCC subtypes. The differences were particularly evident in clear cell RCC with deficient expression for hMLH1 in 92% and for hMSH2 in 61.5% of cases. Loss of both hMLH1 and hMSH2 was also observed to a greater extend in papillary and chromophobe RCC cases than described by Bayiee and Banner [38]. However, a potential role of hMLH1 and hMSH2 as diagnostic or prognostic biomarkers remains uncertain. Apart from a high frequent loss of expression for hMLH1 in clear cell RCC, both markers are not sufficient to differentiate clearly between any of the three subtypes, and expression of hMSH2 – as a potential prognostic marker – is actually limited to advanced tumor stage but not related to metastatic disease.

The impact of MSI on RCC was repeatedly assessed in the literature. Using the NCI consensus marker panel, we detected 2.0% positive cases, confirming former studies which also reported a low incidence, i.e. 0–0.04% of MSI in RCC [16, 19]. In contrast, two former studies not using the NCI consensus marker panel identified 22.7–25% of MSI-positive cases [13, 39]. Our significant correlation of MSI-positive cases and high UICC stage has to be regarded very cautiously, as the number of positive cases was low (n = 3) and there was no correction for multiple testing.

The role of EMAST in tumors currently remains unclear and needs further examination [40]. We did detect EMAST in only one tumor (0.8%). Although our group has already demonstrated that EMAST and MSI on mono- or dinucleotide repeats usually do not overlap, this EMAST-positive case was also MSI-H and MMR deficient. However, due to the low incidence of EMAST, our finding should be interpreted carefully and rather be classified as an accidental result.

Similar to Rubio-del-Campo and colleagues [21], we also found a high amount of LOH RCC cases, which was in accordance with common subtype-specific gains of chromosomal regions. Moreover, we detected LOH in additional regions that might be more uncommon, especially concerning clear cell and papillary RCC.

In summary, our findings support the hypothesis that RCC is a genetic instable disease, but not due to either type of MSI. Further investigations are required to elucidate the pathways of activation and inactivation of MLH-1 and MSH-2 in terms of malignant transformation and whether diminished MMR protein expression might contribute to the different biological behavior of the three main RCC subtypes.

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