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Analysis of DNA Mismatch Repair Gene Expression and Mutations in Thyroid Tumours

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Abstract. *Alterations of DNA mismatch repair genes, primarily demonstrated in hereditary nonpolyposis colorectal carcinomas, were reported to be of relevance for the progression of several sporadic tumours. In this study, the expression and mutations of MLH1, MSH2, PMS1 and PMS2 in a panel of thyroid tumours, including nodular hyperplasia, follicular adenomas and carcinomas, were investigated. The expressions of MLH1, MSH2 and PMS1 were generally higher in malignant tumours than in benign lesions ($p<0.01$). This observation can find potential diagnostic application in the differentiation of follicular adenomas from follicullar carcinomas of the thyroid. No point mutations in the DNA mismatch repair genes MSH2 (exon 12, 13) and MLH1 (exon 15, 16) were found.*

DNA mismatch repair has been shown to play a very important role in the prevention of genetic instability. The defects of this system were first described in HNPCC (hereditary nonpolyposis colorectal carcinoma) (1-7) and later were also demonstrated in several sporadic tumours including melanomas, bladder cancer and breast tumours (8-12).

For humans, four genes, namely MLH1, MSH2, PMS1 and PMS2, are responsible for the control of DNA mismatch repair. It is possible to immunohistochemically investigate the expression of these genes at the protein level. Thyroid tumours have not been investigated in terms of the state of the DNA mismatch repair proteins. Microsatellite instability and some LOH have been found in thyroid carcinomas (13, 14). Accepting microsatellite instability as the result of malfunctioning DNA mismatch repair, a working hypothesis that some alterations of DNA mismatch repair protein expression and eventual mutations in DNA mismatch repair genes are found in thyroid carcinomas can be formulated. Deletion of the short arm of chromosome 3, including the locus of MLH1, reported for thyroid carcinomas, was an additional argument in favour of this hypothesis (15). The overall target of this study was to check our working hypothesis and additionally to judge the diagnostic relevance of DNA mismatch repair gene expression.

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Materials and Methods

Patient cohort. The material investigated consisted of 51 nodular hyperplasia, 32 follicular adenomas, 26 follicular carcinomas, 27 papillary carcinomas and 26 follicular variants of papillary carcinomas. Among the patients were 129 females and 33 males, whose age ranged between 19 and 78 years, averaging 49 years. The patients were diagnosed at the Center of Pathology, University of Göttingen (Germany) and Krakow (Poland) during the years 1998-2003.

Immunohistochemistry. The following antibodies were applied to demonstrate the expression of DNA mismatch repair genes:
a) N-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the terminus of hMSH2 of human origin (Santa Cruz Biotechnology Inc., Heidelberg, Germany).
b) C-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the carboxy terminus of hMLH1 of human origin (Santa Cruz Biotechnology Inc., Heidelberg, Germany).
c) K-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the amino terminus of hPMS1 of human origin (Santa Cruz Biotechnology Inc.).
d) C-20, rabbit polyclonal antibody against the epitope corresponding to an amino acid sequence mapping at the carboxy terminus of hPMS2 of human origin (Santa Cruz Biotechnology Inc.).

The immunohistochemical reactions in the paraffin-embedded tumour tissue were carried out using the Stravigen Multilink kit (Biogenex Laboratories, Hamburg, Germany). The histological sections were mounted on lysine-coated slides. They were deparaffinated using xylol and then transferred to a descending alcohol series and rinsed with distilled water. Before incubation with the primary antibodies, the sections were heated for 10 min on a hot-plate (85˚C) in citrate buffer (pH 6). Afterwards, incubation with the primary antibodies was carried out overnight at 4˚C at an antibody concentration of 1:50. The histological specimens were then rinsed with Tris buffer solution and incubated at room temperature with link (Stravigen Multilink, Biogenex Laboratories) for 45 min. After the detection reaction had been performed using a label (Stravigen Multilink, Biogenex Laboratories) in combination with chromogen fast red (Biogenex Laboratories), the nuclei were counterstained with haematoxylin. In the control reactions the primary antibodies were omitted. The sections were evaluated using the CAS200 image analysis system and the results were expressed as indices indicating the percentage of immunolabelled cells.

PCR-analysis of MLH1 and MSH2 exons. The analysis of mutations in MLH1 and MLH2 was performed in 30 paraffin-embedded lesions of the thyroid (six nodular hyperplasia, six follicular adenomas, six follicular carcinomas, six papillary carcinomas and six follicular variants of papillary carcinomas).

The histological material was cut into sterile Eppendorf microfuge tubes. After washing once with xylene and twice with ethanol (96%) to remove the xylene residue, the cells and cellular debris were obtained by centrifugation at 500 rpm and 4˚C. DNA was isolated from the samples with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Two hundred nanograms of extracted DNA were used for PCR performed on the thermal cycler model 480 (Perkin Elmer, Weiterstadt, Germany). Each PCR was performed on a 50-μl mixture, in a thin-walled test tube, consisting of 1 unit Tfl DNA polymerase, polymerase buffer (BIOzym, Hessisch Oldendorf, Germany) and 20 μM of each dNTP. The mixture was overlaid with mineral oil (Sigma, Munich, Germany). The PCR primers were synthesized by MWG (Ebersberg, Germany) and added at a final concentration of 40 pmol/l per assay. Standard precautions against cross-contamination were taken.

The presence of nondegraded DNA in the samples was tested by semi-nested PCR specific to the human β-globin gene. β-Globin DNA was amplified using the primer pairs 5'-ATGGTGCACCTG ACTCCTGAGG-3' (sense 1)/5'-GCCATCACTAAAGGCACCGA GC-3' (anti-sense) and 5'-CTGTGGGGCAAGGTGAACG-3' (sense 2)/ anti-sense. For amplification, test tubes were heated to 95˚C for 7 min, followed by 40 cycles at 95˚C for 45 sec, 60˚C for 20 sec and 72˚C for 1 min and a final extension at 72˚C for 7 min. For the second PCR run, 1-2 μl of the first run PCR product were used. The PCR conditions in run one and two were identical.

Only β-globin-positive cases were used for nested PCR analysis for the MLH1 and MSH2 exons. Exon 12 of MSH2 was amplified with the help of the following primers: 5'-TTTCTGTTTTTAT TTTTTTACAGG-3' (forward primer), 5'-AAACGTTACCCCCA CAAAG-3' (reverse primer). For the analysis of exon 13 of MSH2 the following primers were applied: 5'-CTAACAATCCATTT ATTAGTAGC-3' (forward primer) and 5'-CATTTCTATCT TCAAGGGACTAGGA-3' (reverse primer). Exon 15 of MLH1 was investigated by applying the primers: 5'-ATTTGTCCCAAC TGGTTGTATCTC-3' (forward primer) and 5'-ACTATACAA TACAGCAACTATCCT-3' (reverse primer). Exon 16 of MLH1 was analyzed using the primers: 5'-GCTTGCTCCTTCATGTT CTTG-3' (forward primer) and 5'-CACCCGGCTGGAAATT TTAT-3' (reverse primer). For amplification of MLH1 exons 15 and 16 and MSH2 exons 12 and 13, test tubes were heated to 95˚C for 7 min, followed by 40 cycles at 95˚C for 45 sec, 60˚C for 20 sec and 72˚C for 1 min and a final extension at 72˚C for 7 min.

For visualisation, 5 μl of each PCR assay were separated on a 2% (w/v) agarose gel containing 0.5 μg ethidium bromide per ml. The gel was registered by means of a CCD camera (Biometra, Göttingen, Germany). The *MSH2* and *MLH1* amplification products were purified with the QIAquick PCR Purification Kit (Qiagen).

A total of 200 ng of isolated DNA were labelled with the PRISM Ready Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions and analysed in an Applied Biosystems DNA Sequencer (model 310). Oligonucleotides previously used for amplification of fragments served as sequencing primers.

Statistics. The data were analysed using a statistical analysis system (SPSS, version 7.5) on an IBM-compatible PC under Windows NT 4.0. They had been previously scanned into a spreadsheet (Microsoft Excel 97), where they were made available to the statistics program *via* an open database connectivity (ODBC) driver.

In general, all indices-related data were rank-scaled, *i.e.*, no means or standard deviations were calculated since the parameters investigated are not metrical data *per se*. The median and other percentiles were calculated for all parameters. Mann-Whitney *U*-tests were used for comparisons between the groups.

Results

Expression of DNA mismatch repair genes in nodular hyperplasias of the thyroid. MLH1-positive cells were found in 16/51 nodular hyperplasias investigated. The MLH1 index did not exceed 5%. MSH2, PMS1 and PMS2 were detected in 12/51 lesions. The median-index of all three markers did not show any positive values. The PMS1 index reached maximally 25%, MSH2-10% and PMS2 5% (Tables I-IV) (Figure 1A).

Expression of DNA mismatch repair genes in follicular adenomas of the thyroid. MLH1 was present in 16/32 adenomas. Its index oscillated between 0% and 20%, reaching a median of 2%. MSH2-positive cells were found in 20/32 tumours. The values of the MSH2 index peaked at 70% and demonstrated a median value of 5%. PMS1 and PMS2 were positive in 7/32 and 14/32 adenomas, respectively. The median expression of both markers did not show any positive values. The PMS1 index reached maximally 30% and the PMS2 index 20% (Tables I-IV) (Figure 1B).

Expression of DNA mismatch repair genes in follicular carcinomas of the thyroid. MLH1-positive cells were detected in 21/26 follicular carcinomas and maximal MLH1 expression

Table I. *Expression of MLH1 in tumours of the thyroid.*

Diagnosis	Positive cases	Percentage of positive cells		
		minimun	median	maximum
Nodular hyperplasia	16/51	0.00	0.00	5.00
Follicular adenoma	16/32	0.00	2.00	20.00
Follicular cancer	21/26	0.00	50.00	90.00
Papillary cancer	13/27	0.00	15.00	90.00
Follicular variants	19/26	0.00	20.00	70.00

Table II. *Expression of MSH2 in tumours of the thyroid.*

Diagnosis	Positive cases	Percentage of positive cells		
		minimun	median	maximum
Nodular hyperplasia	12/51	0.00	0.00	10.00
Follicular adenoma	20/32	0.00	5.00	70.00
Follicular cancer	26/26	0.00	4.75	90.00
Papillary cancer	20/27	0.00	30.00	95.00
Follicular variants	14/26	0.00	10.00	70.00

Table III. *Expression of PMS1 in tumours of the thyroid.*

Diagnosis	Positive cases	Percentage of positive cells		
		minimun	median	maximum
Nodular hyperplasia	12/51	0.00	0.00	25.00
Follicular adenoma	7/32	0.00	0.00	30.00
Follicular cancer	8/26	0.00	0.00	40.00
Papillary cancer	4/27	0.00	0.00	15.00
Follicular variants	19/26	0.00	10.00	60.00

Table IV. *Expression of PMS2 in tumours of the thyroid.*

Diagnosis	Positive cases	Percentage of positive cells		
		minimun	median	maximum
Nodular hyperplasia	12/51	0.00	0.00	5.00
Follicular adenoma	14/32	0.00	2.00	20.00
Follicular cancer	9/26	0.00	0.00	40.00
Papillary cancer	6/27	0.00	0.00	10.00
Follicular variants	23/26	0.00	10.00	40.00

reached 90% of the tumour cells. MSH2 was present in all cases investigated and its index showed high values with a maximum of 90%. PMS1 and PMS2 were found in 8/26 and 9/26 cases, respectively, and maximal index values for both markers reached 40% (Tables I-IV) (Figure 1C).

Expression of DNA mismatch repair genes in papillary carcinomas of the thyroid. MLH1 was found in 13/27 cases and the MLH1 index ranged between 0% and 90%. MSH2 was present in 20/27 carcinomas and demonstrated highly positive index values reaching maximally 95%. PMS1- and PMS2-positive cells were found in 4/27 and 6/27 tumours, respectively. The indices of both markers did not show high values and peaked at 15% for PMS1 and 10% for PMS2 (Tables I-IV).

Expression of DNA mismatch repair genes in follicular variants of papillary carcinomas. Nineteen out of 26 cases contained MLH1- and PMS1-positive cells. The MLH1 index demonstrated values between 0% and 70%. The PMS1 index reached a maximum 60%. MSH2 was present in 14/26 carcinomas with maximal expression of 70% of tumour cells. PMS2 was detected in 23/26 tumours and the PMS2 index values oscillated between 0% and 40% (Tables I-IV).

Comparison of DNA mismatch repair gene expression in nodular hyperplasias and follicular adenomas of the thyroid. A comparison of DNA mismatch repair gene expression in nodular hyperplasias and follicular adenomas of the thyroid demonstrated a highly significant difference in terms of the MSH2 expression ($p<0.01$) and a significant difference for MLH1 expression ($p<0.05$).

Comparison of DNA mismatch repair gene expression in follicular adenomas and carcinomas of the thyroid. Follicular adenomas and follicular carcinomas of the thyroid differed very significantly with respect to MSH2, MLH1 and PMS1 expression ($p<0.01$).

Comparison of DNA mismatch repair gene expression in follicular carcinomas and follicular variants of papillary carcinomas of the thyroid. A comparison of follicular carcinomas and follicular variants of papillary carcinomas of the thyroid demonstrated highly significant differences in MSH2 and MLH1 expressions ($p<0.01$). The difference in PMS2 expression also reached the level of significance ($p<0.05$).

Comparison of DNA mismatch repair gene expression in papillary carcinomas and follicular variants of papillary carcinomas of the thyroid. Papillary carcinomas of the thyroid differed from their follicular variants concerning the expression of all four genes investigated. The difference in MSH2 and PMS2 expressions was highly significant and the difference in MLH1 and PMS1 positivities reached the level of significance.

Comparison of DNA mismatch repair gene expression in minimally invasive and widely invasive follicular carcinomas of the thyroid. The comparison of minimally invasive (n=10)

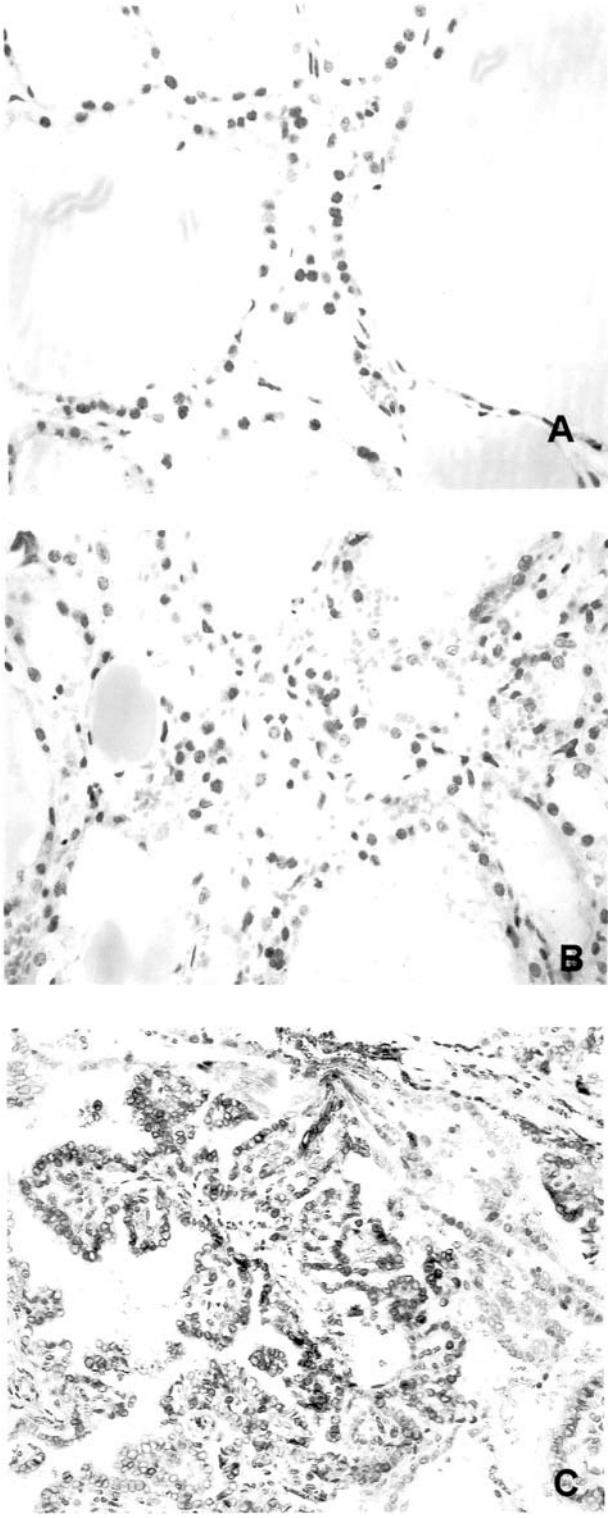

Figure 1. *A) MLH1 immunoreactivity in nodular hyperplasia of the thyroid (x400). B) MSH2 immunoreactivity in nodular adenoma of the thyroid (x400).C) Expression of MLH1 in follicular carcinoma of the thyroid (x400).*

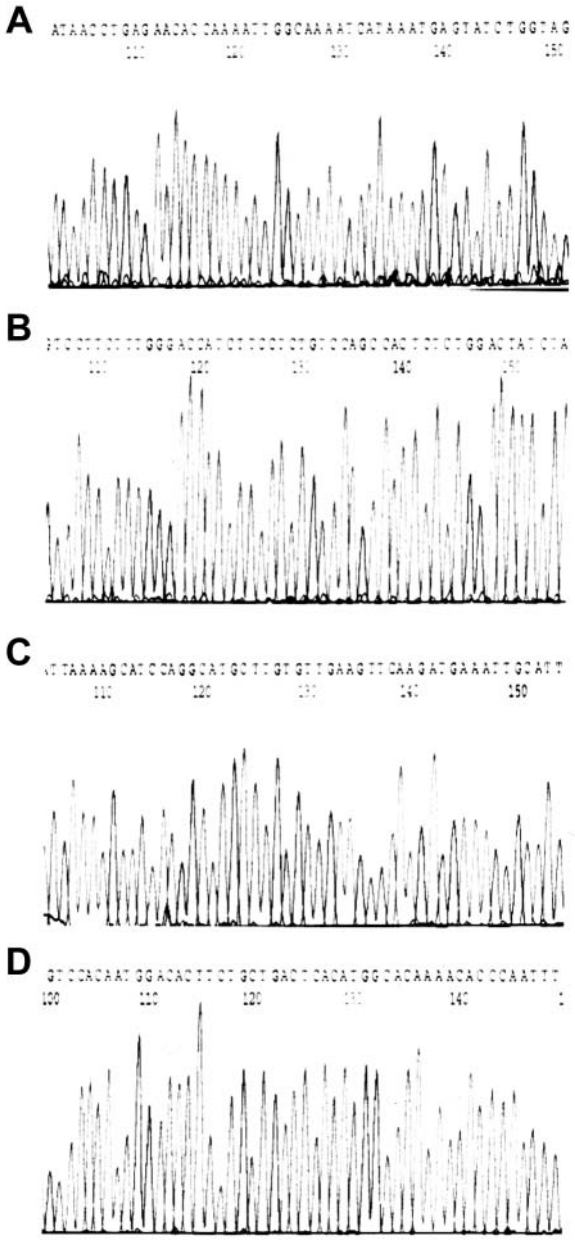

Figure 2. *A) Sequencing analysis demonstrated a normal sequence of exon 12 of MSH2. B) Sequencing analysis demonstrated a normal sequence of exon 13 of MSH2. C) Sequencing analysis demonstrated a normal sequence of exon 15 of MLH1. D) Sequencing analysis demonstrated a normal sequence of exon 16 of MLH1.*

and widely invasive follicular carcinomas of the thyroid did not demonstrate any significant differences with respect to DNA mismatch repair gene expression between both groups ($p>0.05$).

Analysis of mutations in MSH2 and MLH1. The sequencing analysis did not demonstrate any point mutations in MSH2 (exons 12 and 13) or in MLH1 (exons 15 and 16) (Figure 2).

Discussion

Various research groups have tried to overcome the limitations of aspiration cytology in diagnosing follicular neoplasms by examining aspirates from adenomas and carcinomas and applying cytophotometry or morphometric methods. Unfortunately, no parameters were found which enable a reliable pre-operative differential diagnosis between follicular adenoma and carcinoma (16-20).

In our study, highly significant differences between these groups were found with respect to the expression of three DNA mismatch repair genes, namely MLH1, MSH2 and PMS1. All three genes investigated showed generally higher expressions in malignant tumours than in benign lesions.

This observation from paraffin-embedded tissues can find practical application in diagnostic cytopathology of follicular neoplasias of the thyroid in distinguishing between a benign adenoma and a follicular carcinoma of the thyroid. In histology, this distinction is based on the identification of invasion of either the capsule or blood vessels. In cytology, differentiation between a benign and a malignant neoplasm is impossible. Follicular adenomas with either macro- or normal follicular structure can not be differentiated from nodular hyperplasia of the thyroid. Microfollicular or trabecular adenomas can not be differentiated from minimally invasive follicular cancer of the thyroid. Cytological specimens from formerly mentioned lesions contain many follicular cells, creating either follicular formations or rarely syncytial sheets as a substitute of a trabecular structure of a tumour. Colloid is generally not present. Such a cellular picture, namely follicular neoplasia, is an indication for surgical procedure. Pre-operatively, a malignant diagnosis can be expected in 25% of such cases. The presence of intranuclear cytoplasmic inclusions has been noted in follicular variants of papillary carcinomas. Occasionally, these inclusions can also be observed in follicular adenomas and carcinomas as well as in nodular hyperplasias after cytostatic therapy.

The concept of oxyphilic follicular neoplasia was created parallel to the concept of follicular neoplasia. These tumours mainly consist of oncocytes containing broad eosinophilic cytoplasm with granular structure. Mitochondrial hyperplasia in these tumours is the equivalent of cytoplasmic differentiation and is connected with decreased immunoreactivity for thyreoglobulin. Oncocytes are frequently multinuclear and contain hyperchromatic nuclei with large prominent nucleoli. Intranuclear inclusions are sometimes present. Anisocytosis and nuclear polymorphy are often observed. Cytologically, an oxyphilic follicular adenoma can not be differentiated from an oxyphillic carcinoma or from an oxyphilic variant of papillary cancer, or sometimes even from regressive changes. About 15-20% of oxyphilic follicular neoplasias are histologically confirmed carcinomas (16-20). In light of these described diagnostic problems, analysis of the DNA mismatch repair gene expression can find potential application. When insufficient slides for immunohistochemistry are available, the levels of RNA-transcripts for these genes can also be evaluated. An example of such an assay was tested by our research group in malignant melanomas (21).

The generally higher expression of DNA mismatch repair genes in malignant tumours than in benign lesions can be explained by the functional activation of DNA mismatch repair genes connected with DNA damage as a result of malignant transformation. Disturbances in DNA mismatch repair result in microsatellite instability and LOH reported previously for malignant tumours of the thyroid (13-15).

In summary, our study demonstrated the altered expressions of MLH1, MSH2 and PMS1 in the progression of follicullar tumours of the thyroid with potential diagnostic applications.

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