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Allelic Imbalance of 7q32.3-q36.1 during Tumorigenesis in Barrett's Esophagus[1]

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ABSTRACT

Malignant transformation of Barrett's esophagus is characterized by three distinct premalignant stages: intestinal metaplasia (MET), low- (LGD), and high-grade dysplasia (HGD). We reported recently an increase in the frequency of loss of 7q33-q35 between LGD and HGD as determined by comparative genomic hybridization (P. H. J. Riegman *et al.,* Cancer Res., *61:* 3164–3170, 2001). Now the 7q32.3-q36.1 region was additionally characterized by allelotype analysis with 11 polymorphic markers in 15 METs, 20 LGDs, 20 HGDs, and 20 Barrett's adenocarcinomas from different patients. Low percentages of imbalance were determined in METs and LGDs, 7% and 10%, respectively, whereas HGDs and Barrett's adenocarcinomas revealed high percentages of loss, 75% and 65%, respectively. This difference in frequency between LGDs and HGDs appeared highly significant: $P = 0.00007$. The majority of imbalances were found at *D7S2439* and *D7S483*, located on 7q36.1. These data suggest that markers from this area can be used as a diagnostic tool in Barrett's esophagus, *i.e.,* to distinguish between watchful waiting and active treatment.

INTRODUCTION

BE[3] is replacement of the normal squamous epithelium with columnar epithelium and generally occurs in the distal part of the esophagus, probably as a result of gastric reflux (1–3). BE is considered a precursor condition for the development of adenocarcinoma. It includes the following stages: MET, LGD, and HGD. Over the past decades, the incidence of BAC has increased rapidly and is most frequently diagnosed in elderly white males (4). These adenocarcinomas have a very poor prognosis because of early metastatic spread and frequent local recurrence (5). Surgery or mucosectomy is recommended in patients diagnosed with HGD because of the high risk of developing BAC (6). Therefore, it is of major importance to search for characteristics in BE that can help to distinguish LGD from HGD. Thus far, many genomic and protein expression aberrations have been reported during malignant transformation in BE. However, this has not resulted in a marker to discriminate between LGD and HGD. We presented recently a genome-wide overview of the DNA copy number changes during development of BAC based on comparative genomic hybridization (7). An increase of the frequency of loss of 7q33-q35 was found between LGD and HGD, suggesting the presence of a possible biomarker within this large genomic region. The latter study was performed on pairs of adenocarcinomas and adjacent dysplasias present in surgical resection specimens. Therefore, we decided to examine the region 7q32.3-q36.1 extensively by allelotype analysis with 11 polymorphic markers of 75 DNA samples from different patients in successive stages of malignant progression in BE.

MATERIALS AND METHODS

Patient Material. For this study, 15 METs, 20 LGDs, and 20 HGDs were selected from different individuals without adenocarcinoma. Histopathological evaluation and grading of dysplasia were performed by two experienced gastrointestinal pathologists. Abnormal and normal tissues (squamous epithelium and inflammatory infiltrate) were collected from the same tissue section and, therefore, subjected to similar experimental conditions. The 20 adenocarcinoma samples were taken from surgical resection specimens. The mean age of the whole group of 75 patients was 62.5 years of age with a male:female ratio of 6:1.

Laser Capture Microdissection and DNA Extraction. Before microdissecting, the 5-μm formalin-fixed, paraffin-embedded sections were deparaffinized using standard methods, stained for 10 s with H&E, and subsequently dehydrated and air dried. Laser capture microdissection was performed on a Pixcell II (Arcturus, Mountain View, CA). The membrane containing the isolated cells was carefully peeled from the cap and submerged for 2 days at 55°C in 50 μl of DNA isolation buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM of EDTA, 1% Tween 20, and 0.1 mg/ml proteinase K. Proteinase K was inactivated by incubation at 95°C for 8 min.

Polymorphic Markers. Polymorphic markers were selected from the National Center for Biotechnology Information[4] and Genome Data Base[5] databanks based on heterozygosity frequency, as well as coverage and flanking the region of interest. The location of the primers was also compared to the draft sequence,[6] which was used in this study. The exact position of the markers is not entirely clear, although the sequence of the markers along the chromosome in both Genome Data Base and draft sequence databases is almost identical. However, the position of some of the markers might change somewhat during the finishing of the human genome sequence. New primers were designed to shorten amplicons for markers >200 bp (Table 1), because larger amplicons can hamper amplification on DNA isolated from formalin-fixed, paraffin-embedded tissue. After testing, 11 markers were found suitable for additional evaluation (Table 1).

PCR and Interpretation. The 15-μl reaction mixture contained 1.5 μl of 10 × AmpliTaq gold buffer, 2.5 mM of $MgCl_2$, 0.2 mM of deoxynucleotide triphosphate, 0.9 units of AmpliTaq Gold (Perkin-Elmer, Wellesley, MA), 1 μl of DNA, 0.05 μCi α[^{32}P]dATP, and 30 ng forward and 30 ng reverse primer. Five-minute denaturation at 95°C was followed by 35 cycles of 30 s at 95°C, 45 s at the appropriate annealing temperature (See Table 1), and 45 s at 72°C. Elongation was achieved by 10 min at 72°C followed by chilling to 4°C. The PCR products were mixed with 13 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol), denatured for 5 min at 95°C, and kept on ice. Then 4 μl of the PCR product was loaded on a denaturing 6% polyacrylamide gel containing 7 M urea and run at 65 W for 1.5–2 h. Gels were dried and radiographed. Autoradiograms were evaluated by visual inspection.

Allelic imbalance was defined as near or complete loss of a band in the affected sample (MET, LGD, HGD, or BAC) relative to the corresponding normal sample. Allelic conservation was defined as the clear presence of both alleles in both abnormal and corresponding normal DNA. All of the other situations were judged as noninformative.

Calculations and Statistics. The percentage of allelic imbalance in the region 7q32.3–7q36.1 was calculated separately for METs, LGDs, HGDs, and BACs. The Fisher's exact test was used for statistical evaluation and compar-

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[3] The abbreviations used are: BE, Barrett's esophagus; MET, metaplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; BAC, Barrett's adenocarcinoma.

[4] Internet address: http://www.ncbi.nlm.nih.gov/genemap/.

[5] Internet address: http://gdbwww.gdb.org/.

[6] Internet address: http://genome.ucsc.edu/.

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Table 1 *Polymorphic markers 7q32-7q36*

Amplicon	T_{an}[a]	bp	Forward 5′—3′	Reverse 5′—3′
D7S2519	57	105	CTTAGGAACTTGTGGTCCAG	TGCTGTGGTGTATCCTGTGT
D7S640	60	114	TGTCTTCCAGCCCACCCC	GCACATCACCAACAACGTCA
D7S500	57	154	CCAGAATTGAAAACTCAGCATT	GTTCCTTAAATTAAAATGCAAGAA[b]
D7S509	57	141	ATAAGGACTCCTGCTATTTCTT[b]	ACATCAAAATTCAGCACTTAAGT[b]
D7S684	60	157	GCACTCCAGCCTGGGTGA[b]	GATGTTGATGTAAGACTTTCCA
D7S498[c]	57	141	AAAGACATGACACAAAAGGG	CCATTAGATTTAGCAATATGGAA
D7S2426	57	122	GGTGGCTTGCCCAGAGC	TTCCAGTGCATCTGTTTCCC
D7S636[c]	55	168	GAGGAGAGACTCAGAATTGG[b]	AGCTTGTGTGGGGTTTCAG
D7S2439	57	159	CAGCAAAAGGTACAGCAATTTC	GTATAACATAGGTTCATCGGC[b]
D7S483	57	140	TCATTAGCCTTGGCAAAATCAA	TTCTCTAACCCTGTCACCAAA[b]
D7S2465	55	157	ACCTGGGCAACAGAGTGAG	CTTCAAAGAGTTTATGCTTATGT

[a] T_{an} = Annealing temperature used in PCR reaction.
[b] New primers were designed to shorten amplicons for markers with amplicons larger than 200 bp, since this might hamper amplification on DNA isolated from formalin-fixed, paraffin-embedded tissue.
[c] PCR was performed using 3 mM $MgCl_2$ instead of the standard 2.5 mM concentration.

ison of percentages of aberrations. $P = 0.05$ (two-sided) was considered the limit of significance.

RESULTS

Allelotyping with 11 polymorphic markers covering and flanking 7q32.3-q36.1, an area of ~30 Mb, was performed on 15 MET, 20 LGD, 20 HGD, and 20 BAC samples isolated by laser capture microdissection of formalin-fixed, paraffin-embedded tissue sections (Fig. 1). Examples of allelic imbalance are shown in Fig. 2.

Only 1 sample of the 15 tested METs showed allelic imbalance resulting in only 7% loss, whereas 2 of 20 LGD samples revealed allelic loss (10% imbalance). The 20 HGDs displayed 15 samples with allelic imbalance, resulting in 75% loss. A similar percentage was observed for the 20 BACs (13 losses; 65% allelic imbalance). A dramatic increase in the percentage of allelic loss is seen between LGD and HGD (Fig. 1). Comparison of the latter percentages revealed a highly significant difference ($P = 0.00007$).

A substantial part of the detected imbalances cover the whole area of interest and are, therefore, not informative for defining the shortest region of overlap. Most imbalances are found at markers *D7S2439* and *D7S483* (Fig. 3), located at 7q36.1. The area between *D7S2439* and *D7S483* showed 2 losses in 20 LGDs *versus* 12 losses in 20 HGDs (Fig. 1; $P = 0.002$).

DISCUSSION

We have confirmed and better defined the presence of a potentially important marker for malignant progression of BE. Furthermore, we have narrowed the area from 30 Mb, *i.e.,* 7q33-q35 in our previous comparative genomic hybridization study (7), to ~2 Mb, *i.e.,* the region between markers *D7S2439* and *D7S483*. It implies the presence

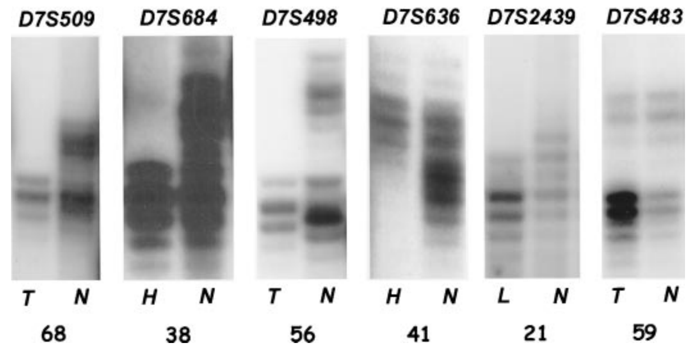

Fig. 2. Examples of allelic imbalance of the used polymorphic markers. Above every panel the polymorphic marker name is given, whereas under the panel the corresponding tissue is shown: *L,* LGD; *H,* HGD; *T,* BAC and *N,* normal. The patient number is depicted at the *bottom* and corresponds to the numbers used in Fig. 1.

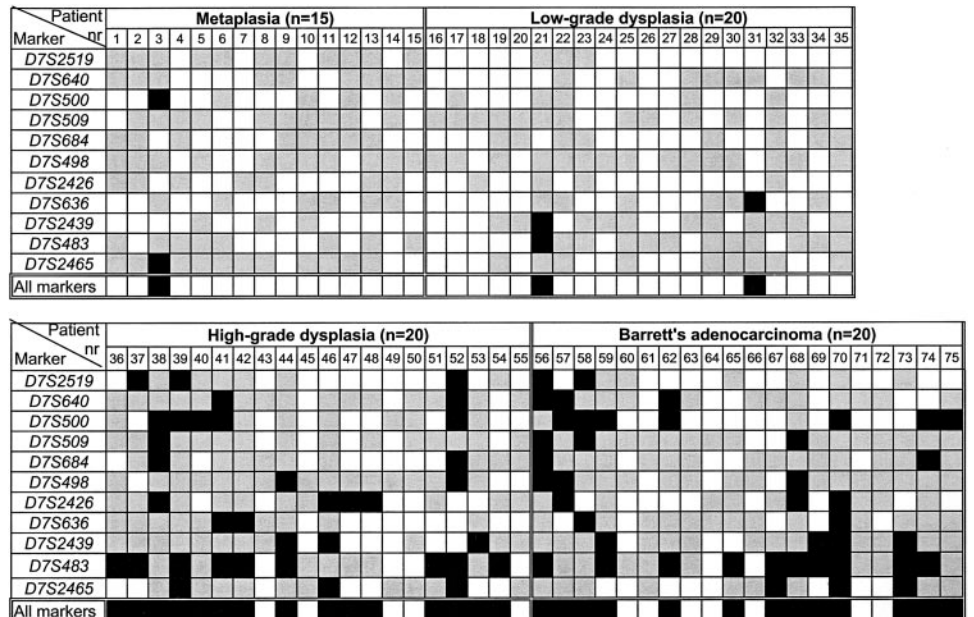

Fig. 1. Overview of allelic imbalances in different stages of malignant progression in BE. ■ represent allelic imbalance; □, conservation; ▨, not informative. The *bottom row* shows the end result for all of the tested markers, *i.e.,* loss or conservation.

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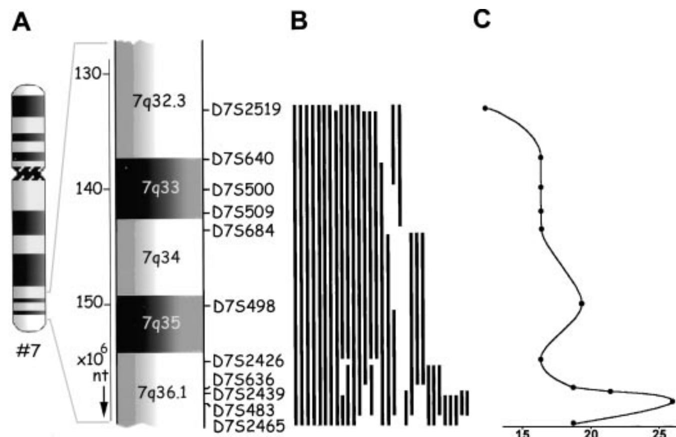

Fig. 3. Overview of the location of the markers and deduced imbalances observed in 7q32.3-q36.1. Locations of the 11 polymorphic markers on chromosome 7 are given by the draft sequence (*A*). The deduced imbalances per sample are represented by *bars* along the chromosome (*B*). The *bars* have been created by extrapolating an imbalance until a conservation is met from another marker in the same sample, thereby considering the noninformative markers as lost. The number of losses of each marker for all samples (MET, LGD, HGD, and BAC) has been determined and subsequently collected in a graphic display (*C*). Note the peak of loss at markers *D7S2439* and *D7S483*.

of a possible biomarker, which, in addition, has tumor suppressive activities. The 7q32.3-q36.1 region has not been reported frequently to be lost in human cancers. Thus far, it has been observed in gallbladder tumors, oral and oropharyngeal epithelial carcinomas, and leukemia (8–10). In gallbladder tumors >60% of allelic imbalance was seen for marker *D7S798*. Interestingly, it is located between *D7S483* and *D7S2465*. We screened the critical area for known genes[7] with tumor suppressive potential and selected two possible candidates. *Caspase 2* is known to stimulate apoptosis and is involved in shedding of intestinal epithelium (11). Loss of these functions could result in uncontrolled cell growth, which is consistent with the biological behavior of HGD (6). However, the position of this gene, proximal in 7q35, is outside the shortest region of overlap we determined. This is in contrast to the second candidate gene, *XRCC2*, which has been reported to be located in a DNA clone together with *D7S483* (12). The latter is within our shortest region of overlap. *XRCC2* is involved in double-strand DNA repair (13). This might not only explain the uncontrolled growth after DNA damage but could also account for the genomic instability in BE resulting in chromosomal changes and aneuploidy. In addition, aneuploidy has been described as a marker for progression in BE (14). There might also be as yet undescribed genes in this area that might turn out to be good candidates. Chromosome 7 is average in gene content, and in the distal part of the q arm some distinct gene density peaks were described (15).

It will be interesting to test the diagnostic value of the distal 7q DNA markers, especially markers *D7S2439* and *D7S483* at 7q36.1, in biopsies of BE patients, who were classified as "indefinite for dysplasia." In this fashion, high-risk patients could be identified without additional delay. A follow-up analysis of our current group was hampered by the fact that the vast majority of patients had been treated after a diagnosis of LGD or HGD. However, we are currently setting up both retrospective and prospective studies to test the diagnostic and prognostic value of loss of distal 7q in BE patients with MET, LGD, or indefinite for dysplasia classifications. Presently, our biomarker could assist in the diagnosis of HGD in difficult cases.

[7] Internet address: http://bioinformatics.weizmann.ac.il/cards/.

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