Aneuploidy Is Associated with TP53 Expression but not with BRCA1 or TERT Expression in Sporadic Colorectal Cancer

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Abstract. Background: Defective expression of genes involved in mitotic chromosome segregation (e.g. AURKA, BUB1B), DNA damage response (e.g. TP53, BRCA1), and telomere function (e.g. TERT) may play a role in the development of tumor aneuploidy. Materials and Methods: The levels of TP53, BRCA1 and TERT were assessed in 55 sporadic colorectal tumors and 37 normal mucosas using tissue microarrays and immunohistochemical detection, and their associations with DNA aneuploidy, levels of mitotic spindle proteins AURKA, AURKB, MAD2L1 and BUB1B and clinicopathological parameters were investigated. Results: DNA aneuploidy was associated only with TP53 alterations. BRCA1 expression in tumors was significantly correlated with individual mitotic spindle protein expressions, and TERT and MAD2L1 expressions were moderately correlated in the tumor group, suggesting a putative role for TERT in MAD2L1 regulation. Conclusion: Loss of TP53 function appears to be involved in the development of aneuploidy, but not in the deregulation of mitotic spindle protein function.

Keywords: Colorectal cancer, aneuploidy, breast cancer 1 (BRCA1), tumor protein p53 (TP53), telomerase reverse transcriptase (TERT), mitotic spindle proteins, aurora kinase A (AURKA), aurora kinase B (AURKB), budding uninhibited by benzimidazoles 1 (BUB1B), MAD2 mitotic arrest deficient-like 1 (MAD2L1).

Materials and Methods

Patient biopsies, clinicopathological features, and clinical outcome. Patient material consisted of 55 tumor resections taken from patients who had undergone attempted radical surgery for sporadic colorectal cancer during the period 1990-2001 at three regional hospitals (Ullevål University Hospital, Asker and Bærum County Hospital).}

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and Vestfold County hospital). The patient group consisted of 30 males and 25 females, with a median age of 68 years (range 35-88 years). None of the patients received radiation or chemotherapy prior to surgery. Twenty tumor specimens contained peritumoral morphologically normal mucosa (proximal normal mucosa). Normal mucosas collected from colectomy specimens without cancer from an additional 17 patients were used as normal controls. The carcinomas were histologically classified as poorly (n=8), moderately (n=38) or highly (n=6) differentiated; 3 tumors showed mucinous differentiation. The degree of tumor spread was assessed using Dukes’ staging: 3 tumors were classified as Dukes’ A, 31 as Dukes’ B, 13 as Dukes’ C and 8 as Dukes’ D. The majority of this tumor series was previously evaluated for ploidy status (5), gross chromosomal aberrations (5), levels of the mitotic spindle proteins AURKA, AURKB, MAD2L1 and BUB1B (22), and levels of proliferation using the Ki-67 antibody (22). Thirty-three (60%) out of the 55 tumors were classified as aneuploid and 22 (40%) as diploid using DNA flow cytometry (5). Forty-one percent, 51%, 53%, and 54% of the colorectal tumors studied in the present work were previously shown to overexpress AURKA, AURKB, MAD2L1, and BUB1B respectively (22). Details of patient survival at last follow-up (January 2007) were obtained from medical records subsequent to having obtained the necessary permission to use patient information according to regional ethical guidelines. Thirty-seven patients were deceased (mean survival time of 33 months) and 18 patients were still alive as of the follow-up date.

Tissue microarrays and immunohistochemistry for BRCA1, TP53 and TERT proteins. Preparation of tissue microarrays was described in detail in our previous report (27). The sporadic colorectal cancer microarrays utilized for the present study consisted of 3 cores of tumor tissue from each patient, 2 cores of proximal normal mucosa, and 2 cores of normal mucosa. All the core tumor specimens had previously been evaluated by an experienced pathologist (OPFC) as representative of the tumor block from which they were sampled. Tissue cores sampled from the proximal mucosa were localized to ≤5 mm from the cancer margin.

Xylene-dewaxed paraffin tissue microarray sections (4 microns thick) were exposed to 0.5% H2O2 solution for 10 min to block endogenous peroxidase, rinsed in tap water for 5 min, and subjected to antigen retrieval in EDTA buffer, pH 8.0 (BRCA1) and in citrate buffer, pH 6.0 (telomerase and TP53) as described previously (27). Tumor sections (as well as the tonsillar tissue biopsies that served as positive staining controls) were subsequently incubated with primary antibodies against telomerase (TRT (H-231), 1:50 dilution; Oncogene Research Products, Cambridge MA, USA) and TERT (1:20 dilution; Cell Signaling Technology, Danvers MA, USA) as described previously (27). Sections stained with Tris buffered saline (TBS) instead of primary antibodies functioned as negative staining controls. TPS3 staining was performed on a Ventana Nexes machine using Ventana “I” view DAB Detection kit (Ventana Medical Systems, Tucson AZ, USA) according to the manufacturer’s protocol. Three to four hundred cells per core were counted for each specimen (cancer, proximal normal mucosa or normal mucosa) for a total of circa 1,000 cells per patient. The mean percentage of positive cells ± standard deviation (SD) or the median percentage (including range) was calculated from the number of positive cells out of 1,000 cells counted. This percentage value (%) positivity defined the protein expression for each sample.

Statistical analyses. SPSS 15 (SPSS Inc., Chicago, IL, USA) and Prism 4 (GraphPad Software, La Jolla, CA, USA) were used for statistical testing and the generation of data tables and frequency distributions. The relationships between protein expression and clinicopathological features were evaluated using parametric or non-parametric contingency testing and correlation analyses as indicated. Overall survival was assessed using time-to-event analyses from the date of surgery to the time of last follow-up and visualized using Kaplan-Meier plots. A p-value <0.05 denoted statistical significance.

Results

Immunohistochemical assessments of BRCA1, TP53 and TERT levels in colorectal tumors and correlations with tumor parameters. Representative images of colorectal biopsies and normal mucosas from tissue microarrays immunohistochemically stained for BRCA1, TP53 and TERT are shown in Figure 1. Protein localizations were primarily nuclear for tumors and normal mucosas, although cytoplasmic staining could be seen in some TERT-stained tumors. For counting purposes, only positive nuclei were counted. Protein expression levels were defined as the number of cells (expressed as a percentage value) positive for a specific protein. Frequency distributions for BRCA1, TP53 and TERT expressions in the tumor group were parametric, non-parametric and non-parametric, respectively, and these were used to determine whether the mean (parametric distribution) or the median (non-parametric distribution) was used as the cut-off level of expression for statistical testing.

Table I shows the mean (BRCA1) and median (TP53, TERT) levels of expression for each of these proteins in normal mucosas, proximal mucosas, and colorectal tumors. BRCA1 expression was not significantly elevated in the tumor group compared to the normal mucosa group and was not associated with tumor DNA ploidy (Table I), nor was it correlated with TP53 or TERT expression (Table II). BRCA1 overexpression (>12% positivity) was measured in 22 out of 53 (41%) evaluated tumors, and was correlated with gain of chromosomal arm 17q21 where the BRCA1 gene is localized (r=0.319, p=0.02). Eleven out of the 55 (20%) tumors had 17q21 gain and 91% of these were aneuploid tumors (p=0.04). Both TP53 and TERT levels were significantly elevated in the tumor group compared to normal mucosas, and TP53 expression (but not TERT expression) was significantly associated with DNA aneuploidy (p=0.01, Table I). TP53 expression showed no correlation with TERT expression (Table II). High TP53 levels (>23% positivity) were measured in 27 out of 55 (49%) tumors. TERT overexpression (>16% positivity) was measured in 26 out of 53 (49%) evaluated tumors, but was not correlated with gain of chromosomal arm 5p15.33 where the TERT gene which codes for telomerase is localized. Seven out of the 55 (13%) tumors had 5p15.33 gain and all were aneuploid tumors (p=0.03). Individually, BRCA1, TP53 and TERT expressions were not significantly
correlated with S-phase fraction (assessed by flow cytometric cell cycle analyses), Dukes’ stage, tumor localization, or with overall patient survival (data not shown).

Correlations of BRCA1, TP53 and TERT levels with AURKA, AURKB, MAD2 and BUB1B levels in the tumor group are shown in Table II. BRCA1 expression demonstrated strong correlations with the individual expressions of all four spindle proteins but was most strongly correlated with Ki-67 levels. BRCA1 expression was not correlated with any spindle protein, nor with Ki-67 in the normal mucosa group (Table III). TP53 expression was not correlated with the expression of any spindle protein, nor with Ki-67 in either the tumor or normal mucosa groups (Tables II, III). TERT expression in the tumor group showed a moderate correlation with MAD2L1 and a trend towards correlation with AURKA expression, but no correlations with any other spindle protein or with Ki-67 (Table II). In the normal mucosa group, TERT was significantly individually correlated to AURKA, AURKB, MAD2L1 and Ki-67 proteins (Table III).

Discussion

The BRCA1 tumor levels were not associated with aneuploidy and were similar to the BRCA1 levels measured for normal mucosa. The findings that 59% of the tumors examined had reduced BRCA1 levels (<12% positivity) was fairly consistent with previously published data for loss of heterozygosity (LOH) at the BRCA1 locus, which has been reported to range from 40% (28) to 49% (28, 29) in colorectal carcinomas generally. BRCA1 overexpression (in the remaining 41% of the tumors) was correlated with gain of chromosomal arm 17q21 where the BRCA1 gene is localized, but was not associated with tumor aneuploidy. Aneuploidy was however clearly correlated with 17q21 gain, indicating that genes other than BRCA1 at this chromosomal locus may play a role in the aneuploidization process, a scenario which will be investigated using array comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH). The BRCA1 protein detected in the present study was most likely wild-type, since BRCA1 gene mutations are known to be infrequent in sporadic colorectal cancer (13). BRCA1 was not significantly correlated with TP53, perhaps not unexpectedly since the high frequency of TP53 mutations (and resultant mutant proteins) that characterize the majority of sporadic colorectal carcinomas might be expected to affect BRCA1 transcription since BRCA1 is a TP53 target gene. Thus it could be expected that BRCA1 is non-functional due to TP53 loss-of-function mutations in the majority of sporadic colorectal tumors (predominantly DNA aneuploid/CIN phenotype). An interesting question is whether BRCA1 is inactivated in colorectal tumors with a wild-type TP53 genotype (predominantly DNA diploid/microsatellite instability (MIN) phenotype), and if so, how. A recent study showed that BRCA1 mutations appeared to be more frequently detected in replication error (RER)-positive (MIN phenotype) sporadic endometrial tumors compared to RER-negative (CIN phenotype) tumors (30), but the situation remains unclear for sporadic colorectal cancer. It would also be interesting to ascertain whether LOH at BRCA1 is more prevalent in DNA diploid tumors, which presumably have functional TP53 compared to DNA aneuploid tumors, the majority of which have non-functional TP53. As LOH at the BRCA1 locus was not assessed in the present work, one can only speculate as to how BRCA1 might be inactivated. BRCA1 was significantly correlated to each of the mitotic spindle proteins examined in the present work in the tumor group but these correlations were absent in the normal mucosa group, suggesting that overexpression of mitotic spindle proteins and BRCA1 inactivation (overexpression) occur together. Several interesting questions include: what impact does overexpression of the individual mitotic spindle proteins have on BRCA1 functionality and does mitotic spindle protein overexpression in and of itself compromise spindle protein function generally in sporadic colorectal cancer? Functional studies are needed to answer these questions and are currently in the planning stage. Loss of AURKA via RNA interference-mediated knockdown was recently shown to result in reduced phosphorylation of BRCA1 and compromised BRCA1 function (25), since AURKA normally binds to and phosphorylates BRCA1 during the G2M transition, but this was not relevant for the present study as overexpression of AURKA was detected in the present tumor material (22).

We and others have previously demonstrated an association between TP53 expression and colorectal tumor aneuploidy (20, 21) and this association was re-confirmed in the present study. The order of events remains unclear, i.e. whether TP53 mutations precede aneuploidy or vice versa. Our recent study of genetic aberrations in sorted diploid and aneuploid fractions from DNA aneuploid colorectal tumors suggested that TP53 deletions do not precede large-scale aneuploidization in colorectal cancer (31), but where TP53 mutations fit into the sequence of events in the progression to gross aneuploidy remains unclear. Since functional TP53 plays an important role in DNA damage response, DNA repair and apoptosis induction in response to apoptotic stimuli, inactivation of TP53 would be expected to contribute to overall genomic instability since tumor cells with abnormal genomes would survive and flourish in the absence of functional TP53. We and others have previously shown that the acquisition of a (mutant) TP53 phenotype is associated with lower spontaneous apoptosis and higher expression of the apoptotic inhibitor B-cell CLL/lymphoma 2 (BCL2) in sporadic colorectal cancer, suggesting that apoptosis is
Figure 1. Representative BRCA1, TP53 and TERT staining in colorectal tumor tissue and normal mucosa. A) BRCA1 staining in tumor tissue; B) BRCA1 staining in normal mucosa; C) TP53 staining in tumor tissue (normal mucosa was negative for TP53); D) TERT staining in tumor tissue, and E) TERT staining in normal mucosa. Original magnification, ×480.
deregulated following loss of TP53 function (32, 33). We have also previously shown that a high number of chromosomal aberrations were associated with a mutated TP53 genotype, suggesting that TP53 mutation and CIN may be linked (5). As was the case for BRCA1, an interesting question is whether overexpression of any of the mitotic spindle proteins contributes to TP53 inactivation in sporadic colorectal cancer. AURKA overexpression leading to (wild-type) TP53 degradation and down-regulation of checkpoint-response pathways (24) would not be expected to be a significant mechanism of TP53 inactivation in sporadic colorectal cancer, since the majority of tumors have TP53 mutations with resultant accumulation/ stabilization of mutant TP53 proteins, which is the major mechanism for inactivation of TP53. This is supported by the present data showing lack of correlation of AURKA and TP53 (predominantly mutant) expressions. However, AURKA overexpression could be a contributory factor to TP53 inactivation in colorectal tumors without TP53 mutations. However, as TP53 mutation analyses were not performed on this tumor material, this cannot be determined, nor can it be concluded with certainty that high levels of TP53 reflect gene mutation in all cases, since they could in some few cases also reflect activation of wild-type TP53 which is involved in the response to genomic stress/DNA damage. In any case, since most of the TP53 proteins detected in the present tumor group are likely to be mutant, AURKA overexpression would have little effect on the degradation of mutant TP53 proteins since they have altered conformations and longer half-lives compared to wild-type TP53. AURKA has also been shown to phosphorylate TP53 at Ser215 leading to abrogation of its DNA binding and transactivation activity (23). Downstream TP53 target genes such as cyclin-dependent kinase inhibitor 1A (CDKN1A) and phosphatase and tensin homolog (PTEN) were shown to be...
inhibited by AURKA in a Ser215 phosphorylation-dependent manner (23). Phosphorylation of TP53 at this residue by AURKA might thus be an important mechanism of inactivating TP53, especially in colorectal tumors that have not otherwise undergone TP53 mutation, and assessment of the phosphorylation status of TP53 at this residue is planned.

Uncapped telomeres resulting from telomere shortening trigger CIN (and aneuploidy) (19) through breakage-fusion-bridge cycles. Such genomic damage leads to cell cycle checkpoint activation and cell cycle arrest in normal cells that do not overexpress TERT (telomerase), whereas most cancer cells overexpress TERT and may also have defective DNA damage checkpoint functions, for example due to inactivation of TP53 or retinoblastoma pathways, such that they may not recognize breakage-fusion-bridge damage as DNA damage or are unable to induce apoptosis. TERT expression was not associated with colorectal tumor aneuploidy, consistent with the overall consensus that TERT expression constitutes a general tumor phenotype, nor was TERT overexpression (in circa 50% of the tumors) correlated with gain of chromosomal arm 5p15.33 where the TERT gene is localized. However, gain of 5p15.33 was associated with tumor aneuploidy, suggesting that genes other than TERT at this chromosomal locus are likely to play a role in the aneuploidization process, as was suggested for 17q21 gain and BRCA1. TERT was recently shown to be transcriptionally regulated by BRCA1, suggesting that BRCA1 is involved in telomere maintenance (34). While a trend towards a weak correlation between TERT and BRCA1 in the tumor group was detected, it did not reach the level of significance; however, this lack of significance does not necessarily imply that the correlation is not biologically relevant. A trend towards a correlation of TERT expression with AURKA expression was seen in the present tumor series, which may be consistent with the reported induction of TERT by AURKA overexpression (26). TERT expression was significantly correlated with expression of spindle checkpoint protein MAD2L1, suggesting that overexpression of this protein may be involved in the regulation of telomerase activity. It has previously been shown that TERT deficiency led to disruption of functional meiotic spindles and misalignment of chromosomes during meiotic division of murine oocytes (35), supporting putative interactions of TERT with one or more mitotic spindle proteins. Intriguingly, the significant correlations of TERT with AURKA and AURKB in the normal mucosa group were lost in the tumor group, suggesting that the acquisition of TERT overexpression in tumors is not involved in and does not affect the regulation of these mitotic spindle proteins.

In conclusion, BRCA1 inactivation is more likely to be the result of TP53 mutation and resultant loss of TP53 function and less the result of overexpression of any particular mitotic spindle protein, despite its strong correlations with all of the spindle proteins examined in the present work. The loss of TP53 function is likely to be a key factor in the emergence of genomic instability since it is permissive for the formation and survival of aberrant (deregulated) colonic mucosal cells.

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