Somatic-genetic aberrations, specific protein levels and their prognostic value in colon cancer
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CHAPTER 3

A substantial proportion of microsatellite-instable colon tumours carries TP53 mutations while not showing chromosomal instability

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ABSTRACT

Chromosomal instability of colon tumours implies presence of numerical and structural chromosome aberrations and is further characterised by absence of microsatellite instability and occurrence of \textit{KRAS} and/or \textit{TP53} mutations. In previously screening 194 colon tumours for both microsatellite instability and \textit{TP53} mutations, we had found 25 microsatellite- instable tumours. In 9 (36%) microsatellite- instable tumours, presumed to be chromosomally stable, we found \textit{TP53} mutations. This prompted us to investigate whether in these microsatellite- instable tumours presence of a \textit{TP53} mutation would be an indicator of chromosomal instability, i.e. whether this would be a category of tumours showing both microsatellite instability and chromosomal instability. For chromosomal instability assessment we performed array-comparative genomic hybridisation analysis of tumour and control DNA extracted from formalin-fixed paraffin-embedded stage III colon tumour specimens. The array consisted of 435 subtelomere-specific BACs. We compared all but one (which gave bad DNA quality) of the microsatellite- instable \textit{TP53} mutation-containing tumours (8) with a similarly sized group of microsatellite- instable tumours without a \textit{TP53} mutation (11).

Microsatellite- instable tumours with a \textit{TP53} mutation showed on average 0.9 aberrations (range 0-3) when assessed with this array system. Those without a \textit{TP53} mutation showed on average 0.7 aberrations (range 0-2). Thus, microsatellite- instable tumours show few chromosomal abnormalities regardless of the \textit{TP53} mutation status. Since in our study microsatellite- stable tumours have on average 3.4 chromosomal abnormalities (range 0-7), a clear difference exists between microsatellite- instable and microsatellite- stable tumours. Because a substantial proportion of microsatellite- instable colon tumours carries a \textit{TP53} mutation while showing relatively few chromosomal aberrations, presence of a \textit{TP53} mutation in these tumours cannot be considered as an indicator of chromosomal instability.

INTRODUCTION

Subdivided according to genomic instability, the large majority of colorectal carcinomas shows chromosomal instability, whereas approximately 15% shows microsatellite instability. The chromosomal- instable phenotype is characterised by aneuploidy, multiple chromosomal rearrangements, absence of microsatellite instability and an accumulation of somatic mutations in oncogenes, such as \textit{KRAS} and tumour suppressor genes, such as \textit{TP53} and \textit{APC} (Fearon and Vogelstein 1990; Lengauer et al., 1997). The microsatellite- instable phenotype is characterised by a near-diploid number of chromosomes and an association with small insertions and deletions mainly in repetitive sequences (microsatellites) (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993;). Many studies have suggested that \textit{TP53} mutations are associated with chromosomal instability (Livingstone et al., 1992; Yin et al., 1992; Agapova et al., 1996; Leslie et al., 2003) and aneuploidy (Carder et al., 1993; Fukasawa et al., 1996; Campomenosi et al., 1998; Clausen et al., 1998). In agreement with this several studies found an inverse relationship between microsatellite instability and \textit{TP53} and/or \textit{KRAS} mutations (Breivik et al., 1997; Salahshor
et al., 1999; Elsaleh et al., 2001; Samowitz et al., 2001). In a previous study, we found, however, that a microsatellite-unstable phenotype and the presence of a TP53 mutation can occur concurrently. In a screening of 194 stage III colon tumours for microsatellite instability and somatic TP53 mutations 25 proved to be microsatellite-unstable (13%). Of these 25 tumours 9 (36%) proved to have a TP53 mutation as well (Westra et al., submitted). The frequency of tumours with TP53 mutations in that study was 53%. The percentages of tumours with microsatellite instability or with a TP53 mutation are in agreement with results in the literature (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993 and 1998; Salahshor et al., 1999; Olivier et al., 2002; Samowitz et al., 2002).

Thus, in these colon tumours a significant degree of overlap was observed between occurrence of a TP53 mutation and of a microsatellite-unstable phenotype. This prompted us to investigate to what extent a microsatellite-unstable phenotype with presence of a TP53 mutation is associated with chromosomal instability. To detect presence of chromosomal instability in the microsatellite-unstable colon cancer cases, for which we knew already the TP53 mutation status, we have used an extended subtelomere-specific comparative genomic hybridisation (CGH) array. We thus compared the degree of chromosomal instability for microsatellite-unstable tumours with or without a TP53 mutation.

MATERIAL AND METHODS

Patient materials

Formalin-fixed paraffin-embedded (FFPE) tumour specimens were available from patients with stage III colon cancer adjuvantly treated with 5-fluorouracil-based chemotherapy. Data about the microsatellite instability and TP53 mutation status are included from a previous study (Westra et al., submitted). The microsatellite instability phenotype was classified according to the Bethesda guidelines (Boland et al., 1998). Tumour and matched normal DNA from all 9 stage III colon specimens, selected for both presence of a TP53 mutation and a microsatellite-unstable phenotype, were analysed and compared with a similarly sized group of 15 microsatellite-unstable tumours without a TP53 mutation. The obtained results were compared with hybridisation results from 16 microsatellite-stable tumours with a TP53 mutation. The types of TP53 mutations found in both microsatellite-stable and microsatellite-unstable tumours were comparable (data not shown). Briefly, after interpretation of the hematoxylin and eosin (H&E) staining from all FFPE specimens, areas with the highest tumour cell content (approximately 70%) were selected and macrodissected (20 µm slices). The QIAamp DNA mini kit (QIAGEN, Westburg, Leusden, The Netherlands) was used for DNA extraction according to the manufacturer’s protocol with some adjustments. These included skipping the xylene treatment, in the final elution step twice putting the eluate (100 µl of preheated (70°C) elution buffer) on the column and incubating it at 70°C for 5-10 minutes before spin down. Prior to labeling and hybridisation extracted DNA’s from tumour and normal specimens were purified using MicroSpin G-50 spin columns (Amersham Biosciences).
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Array-CGH

Our subtelomere array consists of 414 autosomal BACs, that all map within 5 Mb from the telomere of each of the chromosome arms, 18 chromosome X-specific BACs, and 3 chromosome Y-specific BACs. For positioning the BACs with respect to the sequence of the human genome we used the July 2003 human reference sequence (UCSC version hg16) based on NCBI Build 34. The number of BACs per autosomal subtelomeric region ranges from 5-18. The array contains control spots, of COT-1 DNA, total human DNA and Drosophila DNA. BAC DNA was amplified by DOP-PCR (Fiegler et al., 2003) and spotted in quadruplicate onto epoxy coated slides (Schott Nexterion, Mainz, Germany), using a MicroGrid II arrayer (Biorobotics, Cambridge, UK). Array slides were pre-treated prior to hybridisation according to the manufacturer’s protocol. Prehybridisation with 500 µg Salmon sperm DNA in Schott Nexterion hybridisation buffer/5% dextran sulphate was performed at 65°C for 2 h. Excess prehybridisation solution was removed by rinsing in distilled water and the slides were dried by centrifugation.

Patient tumour DNA and matched normal DNA, or a mix of DNA samples from normal FFPE specimens as reference when no matched normal DNA was available, was labelled with Cy3 and Cy5, respectively, using Invitrogen’s BioPrime random labeling kit. After an overnight incubation at 37°C, unincorporated nucleotides are removed by use of the MicroSpin G-50 spin columns (Amersham Biosciences). Tumour DNA and reference DNA were combined on a microcon YM30 column and subsequently dissolved in 50 µl Schott Nexterion hybridisation buffer/5% dextran sulphate, supplemented with 200 µg human COT-1 DNA and 1 mg yeast tRNA. Labelled DNA was denaturated at 99°C for 10 min and stored at 65°C prior to hybridisation. Hybridisation was performed under lifterslips in humidified chambers at 65°C for 48 h. Posthybridisation washes included wash in 2*SSC/0.2%SDS at 65°C for 15 min, followed by a wash in 2*SSC at room temperature for 5 min, and wash in 0.2*SSC also at room temperature for 5 min. Slides are rinsed briefly in distilled water and dried immediately by centrifugation.

Image analysis and processing

Arrays were scanned using the Affymetrix 428 scanner (Affymetrix), and the Jaguar Software package (Affymetrix). The resulting images were analysed with Imagenex software package 5.0 (BioDiscovery inc.). Data were further processed with specifically designed data-analyses software.

Briefly, spots were eliminated if the absolute intensity of the reference (normal tissue DNA) sample was less than two times the average signal of a set of control spots consisting of Drosophila DNA. Raw tumour/reference ratios are calculated for all spots without background subtraction. A normalisation was carried out for each subarray separately, assuming that the median ratio of all spots will be “1”. A spot was eliminated if it differed more than 20% from the median ratio of all replicated spots containing the same BAC. BACs for which only one data-point (spot) was left were also eliminated. For the remaining replicate spots of the BACs the average ratio was then calculated.
An analysis was rejected completely if less than 50% of all BACs met the above-mentioned criteria or if the standard deviation of all BACs was larger than 0.25. We have calculated the mean ratio of all BACs for each autosomal subtelomere. A subtelomere was only assigned a mean ratio if at least 3 individual BACs remained after application of all the criteria mentioned.

**Selection of abnormal BACs per subtelomere**

In principle all BACs per subtelomere that show a mean ratio of \( \geq 1.20 \) or \( \leq 0.83 \) (=1/1.20) between the signal intensities were selected as possibly aberrant. Subsequently the mean ratio and the standard deviation of all remaining modal BACs/subtelomeres (without the presumed aberrant subtelomeres) were calculated. When the first criterion was met, i.e. a mean signal ratio of \( \leq 0.83 \) or \( \geq 1.20 \), a second criterion was applied, implying that the signal ratio for the aberrant BACs per subtelomere should be more than 1.5 standard deviations from the mean signal ratio of all modal BACs.

A gain was determined as having a mean signal ratio of the BACs per subtelomere of \( \geq 1.20 \) and \( \geq 1.5 \) standard deviation above the mean signal ratio of all modal BACs. A loss (deletion) was determined as having a mean signal ratio of the BACs per subtelomere of \( \leq 0.83 \) and \( \leq 1/(1.5 \) standard deviation above the mean signal ratio of all modal BACs), representing the reciprocal of the gain-definition.

**FISH**

Using these arrays, fold changes of the complete set of chromosomes cannot be determined. Therefore, we performed a FISH analysis on the tumour specimens, using centromeric probes for chromosomes #8, #15 or #16 and #1 (pjm 128 (D8Z2); D15Z1; pSE16 and pUC1.77, respectively). All probes were labelled by standard nick-translation with biotin-16-aUTP (Roche, Basel, Switzerland). A selection of 15 tumour samples has been analysed, 5 with a microsatellite-unstable phenotype without a TP53 mutation and 5 showing both the microsatellite-unstable phenotype and a TP53 mutation and 5 with a microsatellite-stable phenotype and a TP53 mutation. From the selected tumour FFPE specimens 4-5 \( \mu m \) slices were made and placed on aminopropyltriethoxysilane (APES, Sigma-Aldrich) coated slides. The pre-treatment of the slides was performed according to Haralambieva et al (2002). Probes and slides were co-denatured at 75\( ^\circ \)C for 10 min in a PTC200 (Peltier Thermal Cycler, MJ Research). Hybridisation was carried out in a humidified chamber at 37\( ^\circ \)C overnight. Excess of probe was washed subsequently, in 4*SSC/0.05% Tween-20 three times at 37\( ^\circ \)C for 5 min, in 0.1*SSC three times at 60\( ^\circ \)C for 5 min and finally in 4*SSC/0.05% Tween-20 at RT for 5 min.

Immunodetection was accomplished by incubating with FITC-avidin (Zymed), biotinylated anti-avidin (Kordia) and again FITC-avidin for 20 min at RT. Between these incubations, the slides were washed in 4*SSC/0.05% Tween-20/5% non fat dry milk (NFDM) three times for 5 min.
RESULTS

Nineteen of the 24 stage III colon cancer specimens with a microsatellite-instable phenotype met the before-mentioned selection criteria for array-hybridisation and thereby gave interpretable results. The specimens included 8 of the 9 cases that had both a microsatellite-instable phenotype and a TP53 mutation and 11 of the 15 cases with a microsatellite-instable phenotype and no TP53 mutation.

Number and type of subtelomeric aberrations of the microsatellite-instable tumours with respect to TP53 status are presented in Figure 1A. Further stratifying by microsatellite instability status and TP53 mutation status shows that the 11 cases with a microsatellite-instable phenotype and without a TP53 mutation have on average 0.7 aberrations, including 0.5 gains and 0.2 losses (median 1.0, range 0-2). The 8 combined cases with both a microsatellite-instable phenotype and a TP53 mutation show on average 0.9 aberrations, including 0.6 gains and 0.3 losses (median is 0.5, range 0-3). For comparison, we also analysed a similarly sized group of microsatellite-stable tumours (Figure 1B). The average number of aberrations for the 13 cases with a microsatellite-stable phenotype and a TP53 mutation as assessed by the subtelomere array is 3.4, including 2.3 gains and 1.1 losses (median 3, range 0-7). The mean numbers of aberrations found in the microsatellite-stable tumours and in the microsatellite-instable tumours are significantly different (3.4 vs. 0.8, respectively, t-test P=0.001). Although small differences are undetectable with our sample set, we are able to detect larger differences. For instance a factor 4 difference in number of aberrations between the microsatellite-instable tumours without a TP53 mutation and those with a TP53 mutation can be detected with a power of 99.5%.

Five out of the 11 (45%) microsatellite-instable tumours without a TP53 mutation showed no subtelomeric aberrations, as did half (4/8) of the microsatellite-instable tumours with a TP53 mutation. Of the 13 microsatellite-stable tumours analysed only 2 (15%) showed no aberrations. None of the 11 microsatellite-instable tumours without a TP53 mutation showed more than 2 aberrations. The same is true for all but one (7/8, 88%) of the microsatellite-instable tumours with a TP53 mutation. For comparison, in the microsatellite-stable tumours 9 of the 13 have 3-7 aberrations.

The chromosomal aberrations that occurred most frequently amongst the microsatellite-stable tumours were gains on 13q (54%) and on 20q (69%). Aberrations on 13q did not occur in any of the microsatellite-instable tumours and an aberration on 20q occurred only once in that group.

Examples of array CGH analyses after data processing, of microsatellite-instable tumours with and without TP53 mutation are shown in Figure 2A; in comparison with a microsatellite-stable tumour with TP53 mutation (Figure 2B).
**Figure 1:** The number and type of subtelomeric aberrations found per specimen, and subdivided according to microsatellite instability and TP53 status.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Subtelomeric Gains</th>
<th>Subtelomeric Losses</th>
<th>TP53 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen 1</td>
<td>10</td>
<td>10</td>
<td>TP53 Mutation</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>11</td>
<td>ND</td>
<td>TP53 Mutation</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>12</td>
<td>10</td>
<td>TP53 Mutation</td>
</tr>
</tbody>
</table>

Black: subtelomeric gains (with mean signal ratio ≥1.20), gray: losses (with mean signal ratio ≤0.83). ND: not determined.

A, microsatellite-unstable specimens with TP53 mutation or without TP53 mutation.

B, microsatellite-stable specimens with TP53 mutation.
Figure 2: Examples of array CGH analyses after processing of data. Normalised intensity signal for the tumour versus normal ratios of the spots/BACs for an example from each of the tumour phenotypes. A, microsatellite-instable with \textit{TP53} mutation and without \textit{TP53} mutation; B, microsatellite-stable with \textit{TP53} mutation. The spots/BACs with a ratio of zero represent the BACs that were excluded after processing of data.
FISH analysis revealed for most tumour sections two signals per nucleus for the centromeric probes used (Figure 3). Small heterogeneous tumour populations with 3-6 signals per nucleus were, however, also seen. These populations, however, never represented more than 5% of the total cell population.

DISCUSSION

In a previous screening of colon tumours for the presence of TP53 mutations, often considered as an indicator for chromosomal instability, and for microsatellite instability, often considered as an indicator for chromosomal stability, we found in 9 out of the 25 microsatellite-instable tumours a TP53 mutation. This prompted us to investigate whether in these microsatellite-instable tumours the presence of a TP53 mutation would indeed be an indicator of chromosomal instability. As many of the chromosomal aberrations occurring in chromosomally-instable tumours will affect the subtelomeric copy number we used the number of subtelomeric regions having undergone a change in copy number as an indicator for chromosomal instability. Using our subtelomere array, we considered presence of more than 2 chromosomal aberrations as chromosomally instable.

We observed chromosomal aberrations (subtelomeric copy number changes) in 10 out of 19 microsatellite-instable tumours, but the number of aberrations was low, only once more than 2 chromosomal aberrations was detected. Among these 10 microsatellite-instable tumours with few aberrations 7 proved to have a somatic TP53 mutation. In the analysed microsatellite-stable tumours, chromosomal aberrations occurred in 85% of the tumours
and the number of aberrations was mostly 3 to 7 per tumour DNA specimen. Thus, a clear difference is observed in the number of chromosomal aberrations between microsatellite-instable and microsatellite-stable tumours.

An association between the occurrence of TP53 mutations and chromosomal instability as shown by Livingstone et al. (1992), Yin et al. (1992), Agapova et al. (1996), Leslie et al. (2003), however, was not seen in our subset of microsatellite-instable tumours where only one of the TP53 mutation carriers is moderately chromosomally instable. Even in our group of microsatellite-stable tumours the correlation between TP53 mutation and chromosomal instability is not 100%, as still 4 out of 13 TP53 carriers appears chromosomally stable (2 or less aberrations). Thus, our results clearly show that presence of a TP53 mutation in microsatellite-instable tumours is not an indicator of chromosomal instability.

Our findings support the results of Curtis et al. (2000) who did not find an association between TP53 mutation status and a CIN phenotype in sporadic colon tumours which had been grown to early passage as subcutaneous xenografts in SCID mice. Also Bunz et al. (2002) and co-workers showed that abnormal p53 function is not sufficient to cause chromosomal abnormalities in CRC tumours. They showed that p53 inactivation by targeted homologous recombination does not give rise to a CIN phenotype in both normal human fibroblasts and human epithelial cancer-derived cell lines. Similar findings were reported in mice studies (Lu X et al., 2001; Toft et al., 2002). The latter study (Toft et al., 2002) showed that MSH2 deficient and p53 deficient mice tumours are diploid. In addition they showed that heterozygosity for p53 in MSH2 deficient tumours resulted in increased MSI and not aneuploidy. However, results from several murine studies contrast these and our findings (Brusa et al., 2003; Murpy et al., 2000; Goepfert et al., 2000). Nevertheless, on the basis of contradictory results it is impossible to conclude to a direct link between p53 and CIN.

According to Leslie et al. (2003) presence of a TP53 mutation is associated significantly with gain on 20q, 13q, and 8q and with loss of 18q. Our study showed that in the microsatellite-stable tumours, which also harbour a TP53 mutation, gains on 20q, 13q, 8q and loss of 18q were present in 69%, 54%, 15% and 0% of these cases, respectively. All cases with a gain on 13q showed a gain on 20q as well. This was also shown by He et al. (2003), who found in all six colon tumours with gain on 13q, also gain on 20q. In the microsatellite-instable cases with a TP53 mutation, however, we found none with gain on 20q, 13q and 8q and only one with loss of 18q. We can, therefore, not confirm association of a TP53 mutation with 20q, 13q and 8q gain in this subset of colon tumours.

Our finding that gain on 20q is overall the most frequently found chromosomal abnormality in stage III CRC is in agreement with the results of He et al. (2003), who found gain on 20q in 83% (10/12) of colon tumours analysed. It has been reported that 20q gain is associated with increased proliferative activity of CRC cells (De Angelis et al., 1999) and that 20q13.2 gains are associated with reduced survival (De Angelis et al., 2001). This seems in agreement with our previous finding that patients whose tumours have a microsatellite-stable phenotype and a TP53 mutation, i.e. those in which 20q gains occur have a shorter survival (Westra et al., submitted). With our array CGH approach we detected the same chromosomal aberrations (mainly 20q and 13q) as reported in literature when using standard metaphase CGH (Rooney et al., 2001; He et al., 2003; Leslie et al., 2003). The frequencies of aberrations which we found were more or less equal to that
reported by Rooney et al (2001), but different from those found by others (He et al., 2003; Leslie et al., 2003). We detected 20q and 13q gains in 31% and 22% of cases, respectively, whereas Leslie et al (2003) found gains in 80% and 76%, respectively and He et al (2003) found gains in 54% and 50%, respectively. These differences might be a result of analysing groups of CRC (colon and/or rectal carcinoma) patients, that were heterogeneous with respect to tumour stage, like by He et al (2003) and Leslie et al (2003). Whereas, we analysed only stage III colon tumours and Rooney et al (2001) included stage III colorectal tumours. Our analysis may underestimate the true number of chromosomal aberrations, since subtelomere-specific arrays will not recognise interstitial gains or losses and tumour cell populations may be heterogeneous and admixed with normal cells. A greater number of aberrations, however, were detected in microsatellite-stable than in microsatellite-instable tumours, which is consistent with the finding of Douglas et al (2004), who used a high-resolution whole genome array instead. This suggests that our small subtelomere-specific microarray may be sufficient for a standardised assessment of chromosomal instability in tumours.

In conclusion we see a clear difference in the number of subtelomeric copy number changes (chromosomal abnormalities) between microsatellite-ine stable (on average 0.8 aberrations), regardless of the presence of a TP53 mutation and microsatellite-stable tumours (on average 3.4 aberrations). A high proportion of these microsatellite-ine stable colon tumours that show only a very low chromosomal instability did carry a TP53 mutation, making clear that TP53 mutation in these tumours cannot be considered as an indicator of chromosomal instability.
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MSI-H TUMOURS WITH A TP53 MUTATION AND CHROMOSOMAL INSTABILITY


Westra JL, Schaapveld M, de Boer JP, et al. Presence or absence of a TP53 mutation better predicts disease-free survival in adjuvantly treated stage III colon cancer patients than microsatellite instability status. (submitted)
