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Aberrations of chromosomes 5 and 8 as recurrent cytogenetic events in anaplastic carcinoma of the thyroid as detected by fluorescence in situ hybridisation and comparative genomic hybridisation

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Abstract Comparative genomic hybridisation (CGH) is a technique which identifies gains and losses of DNA sequence copy number in tumours. We used CGH to search for genetic changes in one of the most aggressive malignancies – anaplastic thyroid carcinoma (ATC). For this purpose, we analysed tumour specimens of nine ATCs and DNA of two ATC cell lines. CGH detected aberrations in 10 of 11 samples, with a mean number of gains or losses per carcinoma of 4.8 (range 0–13). Total or partial changes of chromosome 8 (*n*=6), including gains or losses of 8p (*n*=6) or 8q (*n*=5) were those detected most frequently. Chromosome 5p was amplified in five cases. Gains in two of three samples were found for 3q, 7p, 11q and 20q. Gains in a fewer number were seen for 1p (1 case), 1q (1), 7q (2), 9q (2), 11p (2), 12q (1), 14 (1), 15 (1), 17q (2), 18p (2), 18q (1), 20p (1), 21 (2), Xp (2) and Xq (2). Losses were less frequent than gains and observed for 1p (2 cases), 1q (1), 2p (1), 2q (2), 3p (2), 3q (1), 4q (2), 6q (1), 9p (2), 9q (1), 18p (1), 18q (1) and Y (2). Examples of analysis of tumour sections and cell lines performed by fluorescence in situ hybridisation (FISH) confirmed the gains and losses found by CGH and detected additional signals for 8q21 in tumour cells in a sample with no gains or losses normally in CGH. The results suggest that aberrations of 5p, 8p and 8q, which are rarely found in differentiated thyroid carcinoma, may play an important role in the development of ATC. Therefore, these chromosomes could harbour gene loci potentially involved in the aggressiveness of neoplastic tumours, as shown in tumours such as in this study for ATC.

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Introduction

Anaplastic carcinoma of the thyroid gland (ATC) is a rare malignant tumour with a most unfavourable clinical outcome (for review [37]). In contrast to papillary and follicular thyroid carcinoma (PTC and FTC), little is known about the aetiology and pathogenesis of ATC. Although morphological and immunological data suggested that ATC develops from pre-existing PTC or FTC [28], it is still debated whether or not ATC starts directly from follicular cells without initial precursor steps [3, 41].

Cytogenetic studies on ATC are beginning to answer these questions. Whereas differentiated thyroid carcinomas (DTC) studied in a large number of cases (for review [34]) revealed alterations of chromosomes 3 and 10 as important steps in the tumorigenesis of FTC and PTC [12, 17, 33, 36], respectively, little is known about cytogenetic alterations in ATCs. Five of seven cases investigated using conventional cytogenetics (CG) to date were cell lines prone to artefacts due to karyotype changes by in vitro culture conditions. Thus, cytogenetic studies of cell lines may be misleading, and it has been suggested that their impact is of limited value [17]. Aberrations found in these few cases included a broad variety of chromosomal imbalances, but no typical pattern has evolved. Moreover, CG is restricted to the analysis of metaphase nuclei only, so that additional aberrations in interphase nuclei might be overlooked. Such an effect has been shown in cases of chronic lymphoid leukaemia (CLL), for example, leading to erroneous results [4].

Analysis of interphase nuclei is of interest in order to obtain more meaningful information about the cytogenetics of ATC. Comparative genomic hybridisation (CGH) is a technique which offers the ability to overcome the limitations of CG mentioned above [20]. The importance and reliability of CGH has so far been substantiated for a variety of solid tumours and haematolog-

Table 1 Clinical and histological data of the patients examined. *n.k.* not known

Patient	Age	Gender	pTNM classification[b]	Histological subtype[c]
1	64	Male	pT4pN1pM0	Giant cell pattern with bone formations
2	79	Female	pT4pN1pM0	Spindle cell pattern
3	72	Female	pT3pN0pM0	Giant cell pattern
4	84	Female	pT4pN1pM0	Spindle cell pattern
5	52	Male	pT4pN1pM0	Spindle cell pattern
6	85	Female	pT3pN0pM0	Giant cell pattern
7	76	Male	pT4pN1pM0	Giant cell pattern
8	37	Male	pT4pN0pM0	Giant cell pattern
9	69	Male	pT4pN1pM1	Spindle cell pattern
CL10	78	Female	–	–
CL11[a]	n.k.	Male	–	–

[a] Cell line
[b] According to the WHO classification
[c] According to Rosai et al. [38]

ical neoplasias (for review [21]), and it has also been shown to be of major interest in a very recently published CGH study by Hemmer et al. [11] dealing with ATC.

Therefore, nine solid tumours and two ATC cell lines were analysed using CGH. The results were compared with those found using CG and to the CGH results of Hemmer et al. [11].

Materials and methods

Tumour samples

Tumour specimens of nine patients, obtained during surgical removal of the ATC, were analysed (Table 1). Haematoxylin and eosin-stained sections of all cases were histologically reviewed by two pathologists in order to ascertain the diagnosis of ATC and to estimate the amount of tumour tissue analysed in the corresponding unfixed specimen. Histological subtypes of ATC, according to the proposals of Rosai et al. [37] and tumour stage based on the World Health Organization (WHO) classification (Table 1), at the time of surgery were primarily defined in tissue samples taken from the complete surgical specimen after gross examination, using additional stainings and immunohistochemistry.

For this study, pea-size tissue samples were available which were divided into two halves. One half was snap-frozen and stored at –80°C until use for CGH. The other half was fixed in neutrally buffered formalin overnight and embedded in paraffin. This procedure was chosen specially to obtain the best possible histological control of tissue at the closest proximity to the tumour tissue used for DNA extraction.

Five of the patients were female and six were male. The mean age was 69.5 years, ranging from 37 years to 84 years. Histological diagnosis of ATC in the youngest patient, aged 37 years, was underlined by negative immunohistochemical staining for thyreoglobulin, pan-keratin (KL-1), HMB 45, S-100, CD 45 (LCA) and E-cadherin and faint positive staining for vimentin. No previous disorders of the thyroid, in particular differentiated carcinomas, were reported for any of the patients examined. In addition, two ATC-derived cell lines were analysed. Cell line 10 [16] was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cell line 11 [26] was kindly provided by I. Koehmle, Würzburg, Germany.

Comparative genomic hybridisation

DNA was isolated by standard procedures using phenol–chloroform extraction following proteinase K digestion [38]. Reference DNA was obtained from lymphocytes of healthy donors. Tumour and reference DNA (1 µg) were differentially labelled in a nick translation using digoxigenin (DIG)- and biotin (bio)-conjugated d-UTP (Boehringer, Mannheim, Germany), respectively, as described previously [1, 43].

Hybridisation was performed according to the protocol of du Manoir et al. [5] with minor changes [43]. In short, normal metaphase spreads obtained as described previously [9] were denatured using 70% formamide/2× saline sodium citrate (SSC) (pH 7.0) at 73°C for 2 min and dehydrated in a series of ice-cold ethanol. DIG-labelled tumour DNA (200 ng), bio-labelled normal DNA (200 ng), and *Cot*1 DNA (20 µg) were denatured for 5 min at 73°C in 10 µl hybridisation solution (50% deionised formamide/10% dextran sulfate/2×SSC), incubated for a further 60 min at 37°C, then applied onto the denatured metaphase slides. These were placed under a coverslip and sealed with rubber cement. Hybridisation was performed for 3 days at 37°C in a humidified chamber.

Three 3-min post-hybridisation washes were carried out in 50% formamide/2×SSC at 37°C and three 3-min washes in 0.1 SSC at 60°C. Samples were stained with fluorescein-labelled avidin and rhodamin-labelled anti-DIG-antibodies for 15 min at 37°C. Thereafter, the slides were washed for 10 min in 4×SSC/0.1% Tween20 at 45°C and embedded and counterstained with 5 µl 4,6-diamidino-2-phenylindole (DAPI) (40 ng/ml) containing antifade *p*-phenylenediamine dihydrochloride (1 mg/ml). The metaphase spreads were then covered with a glass coverslip.

Image acquisition and analysis were performed as previously described [43]. Ratio profiles were calculated for at least ten metaphase spreads. The diagnostic thresholds for defining gains and losses of tumour and reference DNA were set at signal ratios of 1.25 and 0.75, respectively. As a positive control, CGH was carried out with DNA of a mammalian tumour cell line (MPE-600, Vysis) with known aberrations together with each hybridisation experiment.

Fluorescence in situ hybridisation

FISH for centromeric regions of chromosomes 1, 2, 3, 7, 8, X,Y (Oncor) and locus-specific probes for 5p12/5q31, 8q21/4 and 13q14 (Oncor) were performed on 4 µm tissue sections mounted on poly-L-lysine-coated slides or nuclei preparations of the two cell lines as described previously [29, 30].

For evaluation of FISH, non-neoplastic tissue sections of at least five patients were carried out to define the thresholds for nullisomic, monosomic, disomic and trisomic counts (data not shown). As recommended by Ward et al. [42], the cut-off level was set at three times the standard deviation added to the mean of signal number, which led to values of 26% for monosomy and 33% for trisomy (data not shown).

Table 2 Comparative genomic hybridisation (CGH) results in specimens obtained directly from the tumour

Patient	Karyotype
1	45, XY, +8m –9*, –18*, +3(q27q29), +17q(q12qter), +20q(cenqter)
2	48, XX, +7*, +8, +12(q14q21.2)
3	46, XX
4	46, XX, +5(cenpter), +7(p22), +8(cenpter), +11(pterq22), –X(p11.3pter)
5	46, XY, +X*, –1(cenpter), –3
6	47, XX, +1(q22q23), –9(cenpter), +8(cenqter), +21
7	46, X, +X, –Y, +5(cenpter), +18(cenpter)
8	48, X0, –Y, +1(p22pter), –2(qcenqter)*, +5(cenpter), +7(pterq31), +14*, +21(p13q21)
9	46, XY, +11(q13)

*Signal ratio did not reach the threshold of 0.75 or 1.25, but passed the values of 0.8 or 1.2

Table 3 Results obtained using comparative genomic hybridisation (CGH) in the two cell lines

Patient	Karyotype
CL10	45, XY, –1*, –2*, +3(cenqter), –4(p21qter), +5(cenpter), –8p(cenpter), +8(cenqter), –9(q21.3qter), +20
CL11	49, XY, –3(cenpter), +3(cenqter), +4(cenqter)*, +5(cenpter), –6(cenq24), –8(cenpter), +8(q23qter), –9(cenpter), +9(q31qter), +11, +15, +17(cenqter), +18, +20(11.2qter)

*Signal ratio did not reach the threshold of 0.75 or 1.25, but passed the values of 0.8 or 1.2

Results

Histology

Histological examination of the ten tumour samples demonstrated an infiltration by the tumour cells of more than 80% of the total cells in seven of nine cases, as shown in case number 8 (Fig. 1a). In one sample (case number 3), tumour necrosis and dense infiltrates of inflammatory cells reduced the amount of tumour cells to approximately 50%. One sample (number 9) showed a tumour cell infiltration of less than 50%. Areas of DTC were not found in any of the specimens analysed.

CGH in tissue

Gains or losses of chromosome 8 were the most frequent imbalances detected in four of nine of the ATC tissues (Table 2, Fig. 3). Two of these four cases displayed a gain of the whole chromosome, whereas one sample showed a gain of only the short arm of chromosome 8. In addition, one sample displayed a loss of the short arm and gain of the long arm (Fig. 2a), similar to an isochromosome. Chromosome 5 was altered in three samples by gains of the short arm (Fig. 1b) as well as the region 7p.

Gains or losses occurring in two samples were found for 1p, 7q, 11q, 21, X and Y. Imbalances in at least one case occurred for 1q, 2q, 3, 3q, 11p, 12q, 17q, 18, 21,9, 11p, 12q, 14, 17q, 18, 18p, 20q and Xp.

One case (number 3) revealed a normal karyotype. As described above, histology of this case showed necrosis and dense infiltrates of inflammatory cells.

CGH in cell lines

In both cell lines, aberrations of chromosomes 5 and 8 were detectable (Table 3, Fig. 3), including the short arm of chromosome 5, and the short and long arm of chromosome 8. In addition, imbalances were found for 1, 2, 3q, 4q, 6q, 9p, 11q, 15, and 20.3p, 3q, 4q, 6q, 9p, 9q, 11, 15, 17q, 18, 20 and 20q.

Fluorescence in situ hybridisation

To find out ploidy of the tumours analysed, FISH was performed for chromosomes shown not to be altered in CGH, using probes for cen1, cen2, cen3, 5q31, cen7, cen8, 13q14 cenX and/or cenY (Table 4 and Table 5). These probes revealed pseudotri- and aneuploid patterns for cell lines CL10 and CL11, respectively. Evidence of tri-, tetra- or aneuploidy was not given in the other samples. FISH was then performed for cen8 and/or 8q21/4 in all samples. In the tissue specimens, FISH confirmed the CGH results with the exception of case number 3, in which necrosis and dense infiltrates of inflammatory cells prevented a reliable CGH. In this sample, additional signals for 8q21 were recognised by FISH in 37% of the tumour cells analysed, whereas inflammatory cells were not found to be aberrant for this locus (Fig. 2b). Although repeated hybridisation attempts were made, FISH for 5p12/5q31 was not successful in formalin-fixed sections.

In cell line CL 10, FISH revealed three signals for 5q31 and 13q14 in the majority of cells, indicating a pseudotriploid pattern, whereas 5p12 detected four or more binding sites in more than 76% of the cells analysed. An elevated signal level relative to that of 5q31 and 13q14 was also detectable for 8q21.

In cell line CL 11, FISH displayed 3–5 signals for 5q31 and 13q14 in 82% and 83% of cells, respectively, indicating aneuploidy of the tumour cells. Again, signals for 5p12 were found more frequently with up to eight signals per nucleus in a remarkable portion of the cells (27%). For 8q24, a similar case was seen, revealing five or more signals in 62% of cells.

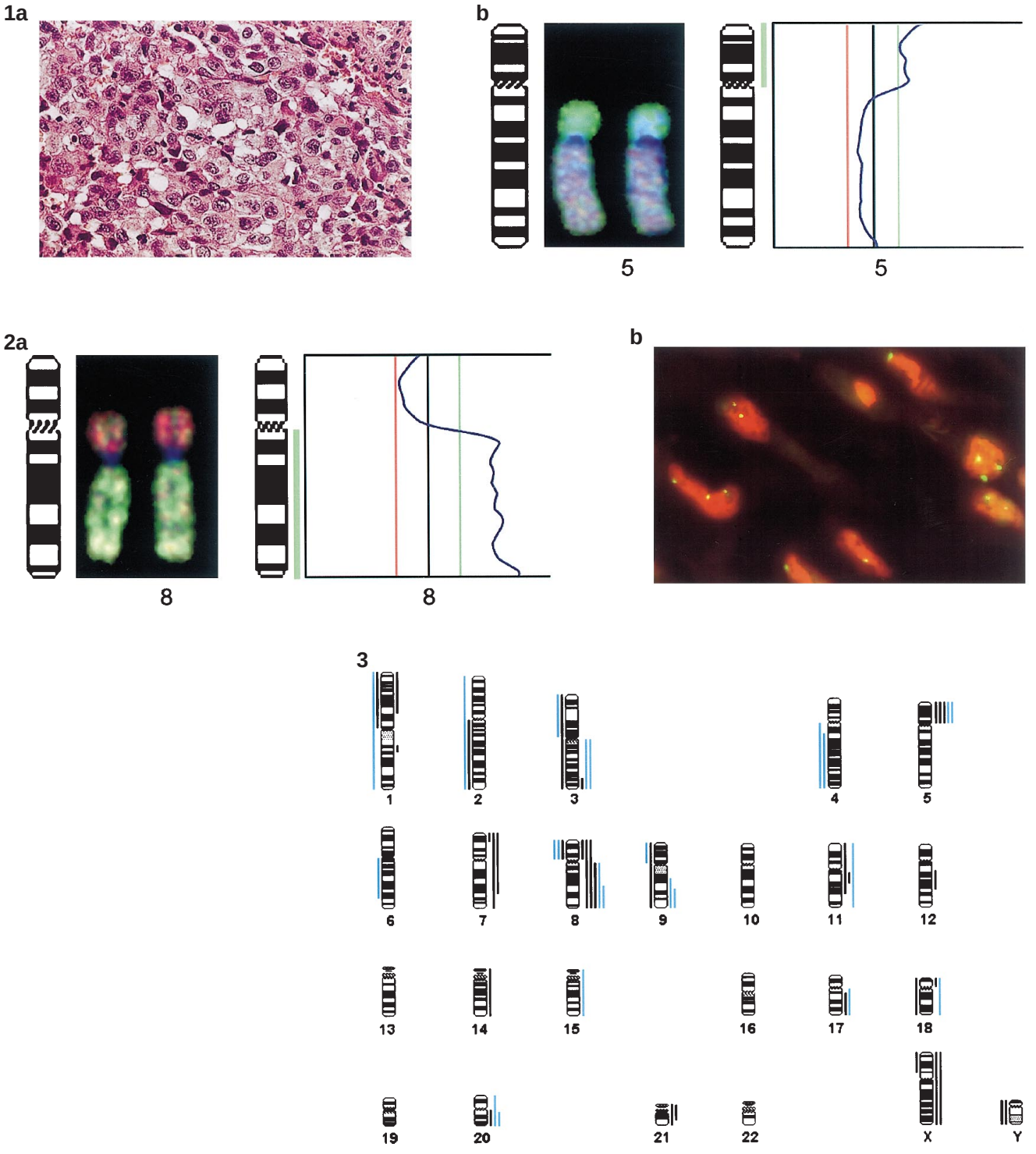

Fig. 1 **a** Histological pattern of anaplastic thyroid carcinoma (ATC) in case number 8, revealing highly polymorphous nuclei and loss of any differentiation. **b** Digital image and signal ratio for chromosome 5 in case 8. The gain of 5p is indicated by the *dark green colour* of the short arm and a *green bar* on the right side of the chromosome ideogram. Signal deviation for 5q did not reach a value of 0.75, as indicated by the red line, and therefore did not define a loss of this chromosome arm

Fig. 2 **a** Gain of 8q in case 6 is evident, whereas the signal ratio for 8p just did not reach a value of 0.75. Since the deviation was clearly detectable and exceeded a value of 0.8, it was assumed to be a loss of 8p. **b** Fluorescence in situ hybridisation (FISH) performed for locus 8q21 in case number 6 confirmed CGH by indicating three and more signals in 37% of cells analysed. Some of the nuclei show less than three or two signals, due to the fact that not all signals in a tissue section can be set in the plane of focus

Fig. 3 Synopsis of the CGH results. Each bar represents an alteration found in a sample. *Black bars* symbolise tumour samples, whereas *blue bars* symbolise the cell lines. Bars on the *left* indicate a loss of genetic material, bars on the *right* indicate a gain for the particular chromosome

Table 4 Results of fluorescence in situ hybridisation (FISH) for chromosomes not be altered in comparative genomic hybridisation (CGH), as well as for numeric and structural aberrations indicated in CGH. Analysis of chromosomes not altered in CGH was necessary to ascertain diploid or near diploid pattern in the tumour cells to set the threshold in CGH at the adequate level

Patient	Locus	Signals per nucleus (%)*				
		0	1	2	3	>3
1	cen1	0	8	92	0	0
	cen8	0	6	43	41	10
	8q21	0	3	42	48	7
2	cen1	1	10	89	0	0
	cen7	0	2	65	33	0
	cen8	0	9	31	43	17
	8q21	0	8	37	42	13
3	cen1	4	6	86	4	0
	cen7	3	8	87	2	0
	cen8	2	7	90	1	0
	8q21	3	4	54	37	2
4	cen1	2	9	88	1	0
	tel8p	6	7	48	39	0
5	cen2	2	17	79	2	0
	cen3	31	62	7	0	0
	8q21	2	6	89	3	0
	cenX	0	5	51	39	5
6	cen2	6	5	89	0	0
	tel8p	15	51	34	0	0
	8q21	0	0	61	37	2
7	cen1	1	16	82	1	0
	8q21	3	14	79	4	0
8	cen7	0	7	46	45	2
	8q21	3	16	74	6	1
	cenX	20	78	0	2	0
	cenY	81	19	0	0	0
9	8q21	0	18	77	5	0
	cenY	5	95	0	0	0

*Values >26% or >33% were defined as aberrant monosomic or trisomic counts

Table 5 Results of fluorescence in situ hybridisation (FISH) in cell lines 10 and 11. In both samples, aberrations of 5p were confirmed as well as for 8q21. Additionally performed FISH for loci not affected in comparative genomic hybridisation (CGH) revealed pseudotriploid pattern for CL 10 and aneuploidy for CL 11. Shifting the threshold for CGH to 0.87 and 1.12 regarding the aneuploidy of the tumour cells did not reveal additional aberrations

	Locus	Signals per nucleus (%)								
		0	1	2	3	4	5	6	7	8
CL 10	5p12	0	0	2	22	59	17	0	0	0
	5q31	0	0	17	72	11	0	0	0	0
	8q21	0	0	11	43	28	18	0	0	0
	13q14	0	0	7	80	11	2	0	0	0
CL 11	5p12	0	0	0	0	0	13	23	37	27
	5q31	0	0	7	38	28	26	1	0	0
	8q24	0	0	2	15	21	46	14	2	0
	13q14	0	0	14	40	25	18	3	0	0

Discussion

Knowledge of cytogenetic changes in ATC has lagged behind comparable information obtained regarding DTC in recent years [34]. Until this year, only two cytogenetic studies have dealt with ATC [17, 23], with only two cases analysed after direct preparation [23]. Other samples were obtained from short-term (n=2) or long-term (n=3) cultures. This gap is now closed by the present study, which adds the cytogenetic analysis of 11 new cases, and the very recently published work of Hemmer et al. [11], giving information about 13 ATC cases.

In the cases of our study, besides numerous other aberrations found in a minor number of samples, three major CG changes evolved with imbalances for 5p, 8p and 8q in up to 6 of 11 cases. These major changes detected in our study are supported by previous observations of aberrations of chromosomes 5 and 8 using conventional CG. These findings were not considered to be of major importance in ATC relative to the other alterations found [17, 23]. Our results, however, are paralleled by another recent study of Hemmer et al. [11] detecting gains of 8q and 5p as well but with a lower frequency of 3 of 13 and 2 of 13 samples, respectively.

In FTC and PTC studies using CG as described, changes of both chromosomes (5 and 8) were also reported, but only as an infrequent singular event (for review [12]). In these studies, the most frequent imbalances involved chromosomes 3 and 10. In two recently published studies [10, 46] using CGH for the detection of karyotype changes in DTC, aberrations of chromosome 1 and, in particular, of chromosome 22 were found in up to 6 of 13 follicular carcinomas [10]. Zitzelsberger et al. [46] detected cytogenetic aberrations of papillary carcinoma secondary to radiation after the Chernobyl disaster using a combined approach of CG, CGH and multicolour spectral karyotyping (SKY). Using these sophisticated techniques, structural aberrations of chromosomes 1, 2, 9, and 13 were seen in up to 19 of 64 cases. These aberrations were listed as the most interesting chromosome regions probably involved in the development of radiation-induced papillary carcinoma beside the known hot spots at 1q and 10q.

Alterations resulting in a loss of 3p material are frequently reported for follicular carcinoma (for review [34]). Our study revealed losses for chromosome 3 only as a total loss of the chromosome and a loss of the short arm, whereas gains on 3q were detected in three cases. This was seen by Hemmer et al. [11], too, reporting a gain of 3q material in two ATC samples. Aberrations of chromosome 10 were not detectable in our ATC samples and seen in one case only in the article of Hemmer et al. [11]. This may be due to the fact that this particular aberration is described mainly as a balanced inversion of the long arm of this chromosome including bands q11–12 [34]. This balanced translocation of chromosome 10 is not detectable by CGH, since the inversion is not associated with gain or loss of DNA but as a rearrangement of the RET proto-oncogene [27, 39]. To overcome this drawback, the application of FISH could be helpful. However, specific probes for this balanced translocation are not yet available.

A study of ATC using CG reported aberrations of chromosome 10q in three of five samples [23]. It may be speculated from our results that aberrations of chromosomes 5 and 8, in addition to those of chromosome 10, typically seen in PTC, are involved in the aggressive biological behaviour and the development of ATC. This assumption could underline the histomorphological finding that areas of DTC are still present in many ATCs [37]. Use of the microdissection technique combined with DNA amplification and CGH would be an interesting approach in such cases to find out whether DTC and ATC share karyotype changes when developing in the same thyroid gland. However, the biopsies in our study were small and did not reveal areas of DTC and ATC in the same sample.

The role that aberrations of chromosomes 5 and 8 play in the development of ATC is not clear. However, these chromosomes have been described to be involved in many other malignancies (for review [25]). 5p is discussed to encode gene $p45^{Skp2}$ [45] and can be altered in epithelial and mesenchymal neoplasms [1, 13, 22, 24]. Alterations of chromosome 8, e.g. 8q, bearing oncogene c-myc located at q24 [21], is correlated to prognostic factors of numerous neoplasms [7, 15, 18, 31, 40]. Trisomy 8 is known to occur in blast crisis of chronic myelogenous leukaemia [2]. Alterations of 8p are also frequently reported and are assumed to be an early event in prostate cancer [8], with commonly deleted regions at 8p23 and 8p12–22 [31]. These regions were found also to be lacking in our study samples.

Interestingly, a recent study by Hemmer et al. [11] detected a gain of 7p as the most frequent aberration, which is seen in our study in 3 of 11 samples. 7p is known to be involved in a variety of malignant tumours and haematological neoplasias similar to chromosome 8 and 8q (for review [25]). However, it is not clear how this chromosomal imbalance is involved in tumorigenesis and how it influences tumour growth.

Another aberration discussed as important in the tumorigenesis of both DTC and ATC [35] is the alteration of the short arm of chromosome 17, bearing suppressor gene p53. However, chromosome 17p was not found to be altered in our samples or in the cases analysed by Hemmer et al. [11]. Allele loss of p53 and a transversion from C:G to G:C in the first base of codon 248 were described by Ito et al. in cell line 11 [16], a small aberration not detectable by CGH. Alterations of structure and/or expression of p53 have been reported for both, DTC and ATC, and the role of this gene in the development of ATC has not been clearly defined [14, 19, 44].

Compared with the results of the previous studies dealing with ATC [17, 23], the number of aberrations in our study and in the study of Hemmer et al. [11] was lower, in particular in the tissue samples. This may be explained due to in vitro cultivation, which was performed in the former studies, leading to additional artificial aberrations [23]. Furthermore, due to the high diagnostic threshold, CGH detects only genetic changes that are present in the major portion of the cells [6]. This was shown for haematological neoplasms, in which both CG and CGH were performed in parallel and whereby CGH revealed a simplified karyotype [43]. The tissues analysed in this study comprised not only tumour cells, but also a small portion of normal cells, probably causing a loss of information in CGH, as seen for case number 3. However, even in cases with only a smaller portion of tumour tissue, CGH detected aberrations as shown in case number 9. As a further impairment of CGH, it has also to be mentioned that aberrations below the sensitivity threshold of 2–5 MB are not taken into account [32].

In conclusion, CGH and FISH revealed frequent alterations of chromosomes 5p and 8 in ATC. These aberrations, which are seen in PTC and FTC as singular events only, seem to be correlated with the histological pattern of ATC and its aggressive biological behaviour. Further studies geared towards the narrowing of the commonly altered regions of chromosomes 5 and 8 may provide new insights into the genes involved and the molecular mechanisms responsible for dedifferentiation of this tumour entity.

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