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Chromosomal aberrations in thyroid follicular-cell neoplasia: in the search of novel  
oncogenes and tumour suppressor genes

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Short Title: Chromosomal changes in thyroid cancer

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**Abstract**

Thyroid cancer derived from the follicular cell is characterised by specific gene alterations that are closely linked to the various pathological types comprising papillary, follicular and anaplastic thyroid cancer. However, the correlation between molecular biology and pathology is not absolute, since about 30% of cases do not harbour the typical gene alterations. This situation, coupled with the demonstration of genetic heterogeneity in thyroid cancer, is a strong motivation for the search of novel gene alterations. Chromosomal aberrations are a good starting point to initiate this search and therefore the current knowledge on chromosomal alterations in thyroid follicular-cell neoplasia is reviewed in this article. An overview on molecular cytogenetic approaches for this strategy is also provided. The identification of novel genetic markers in thyroid cancer will be further improved by integrative approaches combining data from genomic and expression analyses with clinical data. This approach is powerful to identify genetic markers as well as new therapeutic targets in follicular-cell thyroid cancer.

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## 1. Introduction: Relationship between molecular biology and pathomorphology in thyroid cancer

Differentiated thyroid cancers derived from the follicular cell can be divided into two main types, papillary and follicular cancers. Papillary thyroid carcinoma (PTC) arises *de novo* from the follicular epithelial cell and is typically composed of papillary structures. Follicular cancers (FC) are morphologically similar to follicular adenomas, which are benign lesions. Evidence of invasion through the capsule into veins or extra-thyroid tissues distinguishes follicular carcinoma from follicular adenoma. Diagnosis is made on a number of pathomorphological features characteristic for PTCs (e.g. grooved pale nuclei that frequently show intranuclear cytoplasmic inclusions and psammoma bodies which are calcified structures found within the tumours) that are lacking in follicular tumours. The diagnosis of PTC depends on the presence of a number of these features, but not all have to be present for a diagnosis of papillary cancer to be made (Al-Brahim and Asa 2006).

In addition to the two main types of cancer derived from the follicular cells, there are a number of subtypes of both PTCs and FCs. These are named on the dominant structural component. Beside the above mentioned classic PTC, the most commonly found in adults is the follicular variant of PTC which is composed of follicular structures but has the nuclear features and psammoma bodies that are typical for papillary cancer. The solid or solid follicular variant of PTC is composed of solid sheets of cells with or without a follicular component. The latter variant shows variable nuclear features but does contain psammoma bodies. There are other, rarer subtypes of PTCs but these will not be further discussed in this review. FCs are subdivided on the basis of their follicular size – micro, normo or macrofollicular, and where there is a lack of follicular lumina and the morphology appears more solid, trabecular. For an excellent comprehensive review of follicular cell neoplasia, rather than just thyroid cancer, the reader is referred to Kondo et al. (2006).

The broad division made on pathomorphological grounds between papillary and follicular cancers is further supported by molecular evidence. PTCs are associated with rearrangements of the Ret and Trk oncogenes and more commonly in adult PTCs, mutations of the Braf oncogene. The region coding the Ret tyrosine kinase has been shown to fuse with a number of different partners. The Ret oncogene is not expressed in follicular epithelial cells, but fusion of the tyrosine kinase domain to genes with a promoter that is

active in the follicular cells and permits Ret signalling through the MAPkinase pathway (Nikiforov 2002). This so-called RET/PTC rearrangement is formed by fusion of the 3' portion of Ret with different partner genes. The commonest rearrangements, RET/PTC1 and RET/PTC3, involve paracentric inversion on chromosome 10 and are termed PTC1 and PTC3 since both partner genes, H4 and Ncoa4 are also located on chromosome 10. Initial studies reported that there was a higher than expected frequency of Ret rearrangement in PTC arising in the population exposed to radiation from the Chernobyl accident, suggesting some Ret rearrangements might be regarded as a marker for radiation exposure (Fugazzola, et al. 1995; Klugbauer, et al. 1995). More recent papers have suggested that there is not a link between radiation exposure and Ret rearrangements since frequent RET/PTC rearrangements have also been observed in sporadic papillary carcinomas from children and young adults with a prevalence of 40 to 70% of cases (Tuttle, et al. 2008). These findings may therefore reflect more the association between the solid morphological subtype with RET/PTC3 rearrangement and the age of the patient at diagnosis, rather than the aetiology of the tumour (Nikiforova, et al. 2004; Thomas, et al. 1999).

However, in contrast to the post-Chernobyl tumours radiation-associated thyroid tumours either as a result of exposure to radiation for therapeutic purposes (Bounacer, et al. 1997) or developed in the atomic bomb survivors (Hamatani, et al. 2008) exhibited most frequently RET/PTC1 rearrangements. Altogether, these findings indicate that Ret rearrangements are not induced in each case by ionising radiation and therefore make RET/PTC as a radiation-specific marker in papillary thyroid carcinomas questionable, at the very least.

It is important to remember that the correlation between molecular biology and pathology is not absolute: in all of the series published so far, a substantial proportion (30%–50%) of papillary cancers do not harbour rearrangements of the Ret gene at all. A variety of different techniques have been used to assess the frequency of Ret rearrangements and, although this may explain the variation in frequency of Ret rearrangements among studies (Zhu, et al. 2003), a large proportion of papillary carcinomas still remain that are negative for Ret rearrangements and for which the alternative molecular pathways need to be identified. Moreover, a few studies have demonstrated Ret rearrangements in benign tumours associated with radiation exposure (Bounacer et al. 1997; Elisei, et al. 2001; Sadetzki, et al. 2004) and those without radiation history (Rhoden, et al. 2006); however, other studies have failed to substantiate these findings (Thomas et al. 1999), adding further uncertainty to

the specific association of Ret rearrangement with papillary thyroid cancer. A recent paper by Port et al. (2007) has suggested a radiation signature consisting of seven genes which are differentially expressed in post-Chernobyl and sporadic PTC. However, caution is urged in the interpretation of this paper because the radiation-associated group was substantially younger than the control group and of a different ethnic origin, and detailed pathological information is lacking. The signature could therefore relate to variables inherent in the design of the study rather than specifically to radiation exposure.

In contrast to the association between the young onset, solid morphology of PTCs associated with Ret rearrangement, mutations in Braf show a different association with morphology. In the case of Braf the association is with older age at diagnosis and with a more classical PTC morphology. The frequency for Braf mutation varies in a number of studies from 36% to 69% in adult PTC (Cohen, et al. 2003; Kimura, et al. 2003; Powell, et al. 2005). The frequency in post-Chernobyl cases (aged under 18 years at operation) is much lower: less than 10% (Nikiforova et al. 2004); and does not appear to be significantly different from that observed in sporadic cases of childhood thyroid papillary carcinoma (Lima, et al. 2004). This finding is perhaps not surprising as Braf and Ret oncogenic alterations appear to be virtually mutually exclusive in the series published thus far. However, it is clear that all cases that are negative for Braf in young onset papillary cancer are not necessarily positive for Ret rearrangement, and that there are as yet unidentified oncogenic changes in these tumours. A novel rearrangement involving inversion of chromosome 7, resulting in fusion of part of the Braf gene with the Akap9 gene has also been described in three papillary carcinomas from young children in Belarus (Ciampi, et al. 2005). However, further studies in age matched cases will be needed to establish whether this is a radiation-specific event or is related to age at diagnosis.

In contrast, FCs show a spectrum of DNA alterations that are different from PTCs. In particular, mutations of the H-ras and N-ras genes have been observed in FCs and follicular adenomas, but rarely in PTCs (Basolo, et al. 2000; Lemoine, et al. 1989; Manenti, et al. 1994; Namba, et al. 1990). The correct pathological assessment of the tumour is important in this distinction, as follicular variant PTCs can be misdiagnosed as FCs in some circumstances. In addition, the variation in frequency of Ras gene mutation in different series may be related to the differing techniques used in these older studies. A second common oncogenic alteration associated with FC and follicular adenoma is the translocation of peroxisome

proliferator activated receptor  $\gamma$  -Ppar $\gamma$ - to the Pax8 gene promoter (Marques, et al. 2002). As observed with PTC, Ras gene mutation and Ppar $\gamma$ /Pax8 are mutually exclusive in FC, suggesting that their major function in FC may be through the same pathway. Interestingly, rearrangements of Ppar $\gamma$  correlated with younger age at diagnosis and Ras mutation with older age at diagnosis, suggesting that as with PTC, age at onset plays a major role in the molecular signature of the tumour (Nikiforova, et al. 2003). There are a number of other differences in gene expression that are associated with follicular cell neoplasia, but as this review focuses largely on alterations in DNA copy number, these are not reviewed here.

Undifferentiated thyroid cancer is termed Anaplastic Thyroid Carcinoma (ATC), and may derive from PTC, FC or a tumour derived from the C cell component of the thyroid, Medullary Thyroid Carcinoma (MTC, Williams 1966). The oncogenic changes found in ATC therefore reflect not only the changes related to the type of differentiated cancer from which it has evolved, but also new changes associated with its loss of differentiated thyroid cell function.

In summary, the various pathological types of thyroid cancer are closely linked to the alteration in DNA sequence and gene expression, and proper interpretation of molecular biological results should be accompanied by detailed pathological information to permit the researcher to compare results between studies. It is perhaps unsurprising that pathomorphology is so tightly linked to molecular biology - after all the morphology of a tumour is a consequence of the expression of the genes within it.

## 2. Characterisation of chromosomal changes by molecular cytogenetic approaches

Genomic rearrangements are common alterations in haematological malignancies and solid human cancers that often represent recurrent and therefore characteristic alterations for particular tumour types (Mitelman 2000; Teixeira 2006, Mitelman database of chromosome aberrations in cancer (2009), <http://cgap.nci.nih.gov/chromosomes/Mitelman>). A *de novo* characterisation of whole genomic alterations can be achieved by molecular cytogenetic approaches such as comparative genomic hybridisation (CGH, Kallioniemi et al. 1993) and chromosome-specific FISH karyotyping (Schrock, et al. 1997; Speicher, et al. 1996; Szuhai, et al. 2000). This enables the identification of genomic copy number alterations (CNA) and structural chromosomal aberrations. A strong advantage of these techniques is that they do not require a prior knowledge of the alterations present in contrast to single- or multiplex-probe FISH techniques which target one or more genomic loci at the same time and can only be applied if genomic alterations such as copy number changes, inter- and intra-chromosomal translocations or inversions are known.

For many decades G-banding of metaphases and subsequent karyotyping was the only approach to detect chromosomal changes. The application was limited by the requirement of an expert with a high level of expertise in cytogenetics, a need for high quality metaphase spreads and relatively low resolution which made smaller alterations undetectable. A vast improvement was attained by the invention of chromosome-specific FISH painting probes that label each chromosome with a specific colour or colour-mix. This makes inter chromosomal rearrangements identifiable by colour junctions of the FISH-painted rearranged chromosome. Three methods have been published for chromosome-specific fluorescence labelling – M-FISH (Speicher et al. 1996), spectral karyotyping (Schrock et al. 1997) and COBRA-FISH (Szuhai et al. 2000) which mainly differ in the way of probe generation, image acquisition and computational classification of chromosomes. The best possible resolution that can be achieved with these techniques is about 2Mb (Speicher et al. 1996) and requires optimized metaphase spreads preparations.

A technique specially designed for scanning whole tumour genomes for genomic copy aberrations such as gains (duplications, amplifications) and losses (hemizygous –and homozygous deletions) is CGH (Kallioniemi et al. 1993). It uses differentially fluorescence-labelled and fragmented reference and tumour DNA which is *in situ* co-hybridised to normal

metaphase spreads. During hybridisation fragments of reference and test DNA compete for complementary binding-sites presented by the normal metaphase chromosomes. The relative amounts of tumour DNA and reference DNA which bind a particular region on the metaphase chromosomes reflect copy number of the according region in the tumour genome. Gained regions are reflected by a tumour to reference fluorescence ratio larger than one and losses by ratios smaller than one. Since conventional CGH requires metaphase spreads the detection of CNA is typically limited to 10-20 Mb (Parente, et al. 1997).

The limitation of the low resolution of metaphase CGH has been overcome by replacing metaphases with DNA microarrays as hybridisation target (Solinas-Toldo, et al. 1997) and is appropriately named Array CGH (Figure 1). In addition to higher sensitivity, this technical improvement has several advantages such as higher potential throughput, and less amount of time from sample to result due to development in computer technology and bioinformatics which is needed to process the type of data produced. Moreover, an expertise of a cytogeneticist is not an absolute requirement. The first generation of DNA arrays for array CGH used large-insert clones such as BACs and PACs (Fiegler, et al. 2003; Pinkel, et al. 1998) giving a detection limit of 1Mb. Even though resolution of BAC arrays using a higher number of BAC clones was steadily increased and the advent of arrays using oligonucleotides as hybridisation targets (Carvalho, et al. 2004) has resulted in complete coverage of the genome with almost no gaps (Fiegler, et al. 2006), a majority of published array CGH studies used 1Mb BAC array CGH. This may be due to the fact that BAC arrays are relatively cheap, robust and applicable to DNA from formalin-fixed paraffin embedded (FFPE) tissue (van Beers, et al. 2006).

Single or multicolour FISH approaches that detect known gene targets can be used on either metaphase preparations or interphase cells of archived tissue samples in order to identify numerical or structural alterations at a single cell level. The labelled DNA from large-insert clones is typically used as a DNA probe for hybridisation on metaphase spreads or on sections from FFPE tissue blocks.

Hybridisation on metaphase spreads enables the researcher to narrow down the position of chromosomal breakpoints of translocations. For this, a series of probes is selected which bind within the region of the suspected chromosomal breakpoint. Subsequent hybridisation identifies either breakpoint-spanning or – flanking FISH probes whose mapping information can be used for the identification of candidate genes from gene databases. The clones

additionally can be used as translocation-specific probes for interphase-FISH on tissue samples. This strategy was used, for example, in human PTC to investigate the frequency and distribution of Ret/PTC rearrangements (Unger, et al. 2004). Moreover, FISH probes can be generated mapping candidate regions of genomic gains or losses in order to confirm results from array CGH studies. Such alteration-specific FISH probes are also a valuable tool to verify on large tumour cohorts (tissue microarrays) and to validate results from cell lines in human clinical tissue collections.

The recently developed next generation sequencing approaches become available to a growing community of researchers and have the potential to give answers to various biological questions by using genomic DNA or total RNA extracted from human tissue or blood. Its main limitation is still the exorbitant costs for running and maintenance of equipment and for carrying out the experiments itself. A review by Mardis (2008) gives an excellent overview about next generation sequencing and its potential in modern molecular biology. Bashir et al. (2008) describe the potential and limitation of the characterisation of chromosomal rearrangements using next generation sequencing. In particular, next generation sequencing is a promising approach for the detection of chromosomal rearrangements in tumours because it is potentially applicable to DNA from both archived and fresh tumours.

### 3. Cytogenetic alterations in thyroid follicular-cell neoplasia

The tumourigenesis of human solid tumours is driven by the aberrant function of genes involved in cell proliferation, apoptosis, genome stability and angiogenesis (Hanahan and Weinberg 2000). Many different factors such as genome copy number changes, structural aberrations, polymorphisms, point mutations and epigenetic modifications can result in an aberrant gene function. Thereby the selection of these mechanisms varies, e.g. inactivation of tumour suppressor genes can be caused by deletion, mutation or gene methylation whilst activation of proto-oncogenes can be achieved by gene rearrangements or gains of chromosomal regions. Thus, chromosomal aberrations are important features of solid tumours with distinctive impact on the function of cancer genes.

This also holds for thyroid follicular-cell neoplasia. The cytogenetic features of these tumours mainly depend on the type of thyroid cancer and partly allow distinguishing papillary, follicular and anaplastic cancers by characteristic genomic alteration patterns. Similar to other solid tumours, much more data on DNA copy number changes is published compared with the amount of data available on structural chromosome aberrations. This is due to the technical fact that identification of structural rearrangements only is feasible from primary short-term cultures but not from archived tumour tissues whilst genomic copy number analysis can be well done from both.

In papillary thyroid carcinomas (PTC), 10q rearrangements with breakpoints at 10q11.2 leading to an activation of the *Ret* oncogene (Santoro, et al. 1992) were frequently found (Pierotti, et al. 1992). Especially in radiation-associated PTC, several translocations have been reported which led to novel *Ret* activating fusion proteins (Corvi, et al. 2000; Fugazzola, et al. 1996; Klugbauer, et al. 1998; Klugbauer and Rabes 1999; Nakata, et al. 1999; Pierotti, et al. 1996; Salassidis, et al. 2000, reviewed by Santoro et al. (2006)). Chromosomal translocations and inversions with breakpoints at 1q22 were shown to activate another receptor tyrosine kinase, *Trk* and, just like *Ret*, was only seen in PTC (Bongarzone, et al. 1996; Said, et al. 1994).

Since only a subgroup of PTC showed the above mentioned aberrations other studies which aimed to identify additional chromosomal abnormalities reported chromosomal breakpoints on 1p32-36, 1p11-13, 3p25-26 and 7q32-36 in PTC after conventional karyotyping (Roque, et al. 2001). In primary cultures of radiation-associated thyroid tumours, novel breakpoints on chromosomes 4q, 5q, 6p, 12q, 13q and 14q were described by comprehensive analysis using

conventional and spectral karyotyping (Zitzelsberger, et al. 1999). Spectral karyotyping also uncovered complex rearrangements in some tumours (Lu, et al. 2009; Weier, et al. 2006) that have been investigated further by a BAC-FISH approach in order to delineate chromosomal breakpoints. In Figure 2 an example for fine-mapping of chromosomal breakpoints by FISH is shown for a case of a post-Chernobyl PTC.

Much more data on DNA copy number changes in PTC are available from which Table 1 summarizes the most commonly cited in the published literature. In a conventional CGH study on radiation-associated PTC the most frequent copy number changes reported were on chromosomes 2, 7q11.2-21, 13q21-22, 16, 20, 21 and 22 (Richter, et al. 2004) which was in accordance with the chromosome 22 changes reported in other studies (Hemmer, et al. 1999; Singh, et al. 2000) linking its presence to a widely invasive subtype of PTC. In addition, Kitamura et al. (2000) described an association of allelic losses of 1q, 4p, 9 and 16q with survival in PTC. More recent studies using array-CGH of specimen enriched for tumour cells by microdissection showed a more common appearance of copy number changes in PTC (Finn, et al. 2007; Unger, et al. 2008). In these studies, recurrent aberrations were reported which are in accordance with previous CGH studies and which discriminate RET/PTC-positive and -negative tumours (Unger et al. 2008) or are typical for wildtype Ret or Braf tumours (Finn et al. 2007). In focal papillary carcinoma with changes in follicular-patterned thyroid nodules trisomy 17 was identified which potentially reflects a specific marker of this subset of thyroid lesions (Frau, et al. 2008). All these data on DNA copy number changes and structural chromosome aberrations in PTC suggest the existence of additional “driver” aberrations beside the known gene alterations of the MAPK pathway (Ret, Braf).

Compared to PTC chromosomal changes are much more frequent in follicular and anaplastic thyroid carcinomas. In follicular thyroid carcinomas (FTC), cytogenetic abnormalities of chromosome 3 are characteristic changes (Herrmann, et al. 1991) and losses at 3p and of 3p25-pter in particular are the most common chromosomal aberrations in FTC (Roque, et al. 1993). Further CGH analysis of FTC defined three common regions of losses at 3p25-pter, 3p21, and 3q29-qter suggesting the involvement of more than one genomic region of chromosome 3 in follicular carcinogenesis. It is known that translocations with breakpoints at chromosome 3p25 lead to rearrangements of the gene Ppar $\gamma$  (Kroll, et al. 2000). Other frequent copy number changes described in FTC are deletions of 1p, 6p/q, 8p/q, 9p, 11q, 13q, 18q, and 22q (Hemmer et al. 1999; Hemmer, et al. 1998; Roque, et al. 2003). In

addition, loss of chromosome 22 was reported to be associated with an invasive phenotype in FTC. This finding supports the idea that an aggressive phenotype in thyroid cancer is driven by the same genetic changes irrespective of the tumour type since chromosome 22 changes were also linked to a widely invasive phenotype in PTC. The oncocytic subtype of FTC commonly shows multiple numerical chromosome changes including polysomy of chromosome 7 (Dettori, et al. 2003) and a new possible mechanism of Braf activation (Ciampi et al. 2005) by gain of copy number. Braf gene reduplication has been shown to be present in these tumours, suggesting that activation of the MAPkinase pathway is also critical in thyroid follicular cell tumorigenesis although Braf mutations or RET/PTC rearrangements are absent (Musholt, et al. 2008).

Anaplastic thyroid carcinoma (ATC) is a rare but particularly aggressive tumour (Chiacchio, et al. 2008). Surprisingly, the extent of DNA copy number changes does not directly correlate with the aggressiveness of tumours, when different histological types are compared. This is supported by the finding that only few ATC with an abnormal karyotype have been reported (Jenkins, et al. 1990; Mark, et al. 1987). More recently, advanced molecular cytogenetic approaches such as spectral karyotyping enabled the detection of numerous structural aberrations in ATC cell lines revealing chromosomal breakpoints at 1p36 and 17q24-25 which are novel in ATC as well as breakpoints at 3p21-22 and 15q26 which were described for well-differentiated thyroid cancers (Lee, et al. 2007). In contrast to this limited knowledge on structural chromosome aberrations in ATC, copy number changes were reported to greater extent. Recurrent chromosomal imbalances are gains of 1q and 20q which both were described in cell lines and in primary tumours (Lee et al. 2007; Miura, et al. 2003). In another study gains and losses on chromosomes 8p and 8q as well as gains on 5p were regarded as typical changes which were rarely found in well-differentiated thyroid cancers (Wilkins, et al. 2000). Overall, an increase in aberration complexity was reported for the progression from well-differentiated to poorly differentiated and anaplastic carcinomas. In contrast to Wilkins et al. (2000) chromosomal abnormalities of 5p15, 5q11-13, 19p/q (gains) and of 8p (loss) were regarded as common to all thyroid cancer types, whereas gains on 1p34-36, 6p21, 9q34, 17q25 and 20q as well as losses of 1p11-31, 2q32-33, 4q11-13, 6q21 and 13q21-31 were found only in poorly differentiated tumours and ATC (Wreesmann, et al. 2002). In this study, recurrent gains of 3p13-14 and 11q13 and loss of 5q11-31 were reported as unique markers for ATC. Although there are coincidental findings

in these reports such as chromosome 20 abnormalities, a common issue with all of the studies on ATC seems to be the low number of cases studied (range: seven to 28 cases per study) which makes it difficult to identify specific recurrent aberrations.

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#### 4. New candidate genes in thyroid cancer derived from chromosomal aberrations

As mentioned above a number of gene alterations are known in thyroid follicular-cell neoplasms. However, the reported gene alterations do not occur in all cases of a particular thyroid cancer type and, moreover, frequently show a heterogeneous distribution of alterations within the tumour (Ciampi and Nikiforov 2007; Unger et al. 2004; Unger, et al. 2006). This was a strong motivation to search for gene alterations in thyroid follicular-cell neoplasia which interact with the known genetic changes or act as alternative drivers of tumourigenesis.

To address this question many studies used gene expression approaches, some of them combined with LOH analysis, for the detection of novel tumour suppressors or oncogenes. In one of these studies this strategy resulted in the identification of the noncoding RNA gene, Nama, which is downregulated in PTC positive for B-raf mutation and associated with growth arrest (Yoon, et al. 2007). Another novel candidate gene associated with ATC is Plk1 (Polo-like Kinase 1) which was identified by expression microarray screening. It represents a serine/threonine kinase playing an important role in a number of G2- and M-phase-related events (Salvatore, et al. 2007). It is of special interest that Plk1 was subsequently shown to represent a potential therapeutic target in ATC (Nappi, et al. 2009). In FTC overexpression of Akt1 and Akt2 as well as increased levels of total AKT activity has been reported (Ringel, et al. 2001). Other components of this pathway such as Pi3kca have been shown to be gained in copy number or mutated in both, FTC (Wu, et al. 2005) and ATC (Garcia-Rostan, et al. 2005). However, Akt activation which is linked to tumour progression (Guarino, et al. 2005; Vitagliano, et al. 2004) also plays a role in PTC tumourigenesis where RET/PTC rearrangements are associated with the expression of genes downstream of PI3K (Giordano, et al. 2005).

Another strategy to discover candidate genes involved in thyroid tumourigenesis is the usage of genomic approaches with chromosomal aberrations as the starting point. There are different molecular cytogenetic approaches to delineate chromosomal breakpoints. One BAC-FISH-based approach allows cloning of aberrations by using pools of uniformly labelled BAC clones covering a relatively large candidate region in the first place followed by a hybridisation with individual BAC probes labelled with individual dyes (Lu et al. 2009). This approach has been applied to a primary culture of post-Chernobyl thyroid tumours PTC in order to characterise chromosomal alterations on chromosome 9 (Weier et al. 2006). In

another study, the Thada gene, a putative death receptor-interacting protein, was identified with the same approach on chromosome 2p21 of a thyroid adenoma cell line (Rippe, et al. 2003). Fig. 2 illustrates FISH mapping of chromosomal breakpoints of an insertion of q-terminal parts of chromosome 7 into chromosome 12 performed on the primary culture of a post-Chernobyl PTC. For this study YAC clones (CEPH library) and BAC clones (RPCI library) were used in FISH hybridisations. The breakpoint-spanning hybridisation probes enabled the identification of the candidate genes Ppp1r9a, Pon1, Pon2, Pon3, Asb4, Pdk4, Dynclil on chromosome 7 and Spryd3, Igfbp6, Soat2, Acat2, Csad, Pcap, Znf740, Itgb7, Rarg on chromosome 12 (personal communication with K. Unger).

Another strategy to narrow down chromosomal alterations is the use of array-based approaches. Depending on the resolution of the arrays used these approaches often require subsequent BAC-FISH fine-mapping of chromosomal regions or breakpoints. There are only few array CGH studies on DNA copy number changes in thyroid follicular cell neoplasia and only three in PTC (Finn et al. 2007; Rodrigues, et al. 2007; Unger et al. 2008). Another study from Lee et al. (2008) focused on ATC whilst Chen et al. (1998) showed data on a mixed collection of tumours (PTC, FTC, ATC). For PTC a variety of novel candidate genes was reported from the most recurrent copy number changes. Finn et al. (2007) described amplified Tp73 (1p36.33), Snrpn (15q12) and Pdgfb (22q13.1) to be present in wild-type B-raf tumours only. In contrast, Unger et al. (2008) showed different copy number alterations for RET/PTC-positive and -negative PTC and reported a panel of candidate genes that are derived from these discriminating regions of copy number changes. For the commonly altered regions they described Rmi1, Gas1, Shc3, Syk, FancC, Ptch1, Klf6, Net1, Rsu1, Nrp1, Pard3, Ret, Anxa7, Slit1, and Timp3 as tumour-related candidate genes that may be additionally involved in tumorigenesis. The discrepancy of the reported candidate genes are presumably due to the fact that different tumour cohorts were investigated in the two studies (childhood vs. adult patients, differences in the genetic background for B-raf mutations and Ret rearrangements). The only study on copy number in ATC so far demonstrated a deletion of 9p21.3 which encodes for cyclin-dependent kinase 2a/p16 (Cdkn2a) and gains of the genes Ubch10 (20q13.12) and Ccnd1 (11q13). The alterations represent the most frequent changes in this series of 27 ATC (Lee et al., 2008). Most of the studies using array CGH validate their findings in an exemplarily manner for selected candidate genes by BAC-FISH. Since array CGH is a genome-wide screening approach which

comes along with the possibility of false-positive findings cross-confirmation of results is indispensable.

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### 5. Future directions: integrative biology performed in the GENRISK-T project

Although genomic alterations are useful starting points for an elucidation of molecular mechanisms in thyroid follicular-cell neoplasias the question remains which changes are crucial in terms of leading to aberrant gene expressions and tumour phenotypes. To address this key question an integrative statistical approach of data analysis is necessary combining data on genomic and expression changes with clinical data on tumour phenotypes and clinical data of patients. Such an integrative analysis is demonstrated in Fig. 3 and is pursued in an EC-coordinated consortium called GENRISK-T which focuses research at different molecular levels on an age- and histology-matched cohort of post-Chernobyl PTC from the Chernobyl Tissue Bank (CTB).

Large scale collections of biological samples of various formats from human thyroid cancer such as those available from the Chernobyl Tissue Bank ([www.chernobyltissuebank.com](http://www.chernobyltissuebank.com)) are changing the way in which research can be done. In the past researchers have had to rely on small scale collections from usually their own institution, and because of financial constraints on research budgets, have been able to analyse the samples with only one molecular biological technique. Research Tissue Banks open up the prospect of different groups of researchers being able to have access to material in different formats, the molecular analyses of which complement each other. The Genrisk-T project provides a paradigm for this type of project. The aim is to identify genes in thyroid cancer, with specific relevance to radiation induced thyroid cancer and to develop animal models that can be used to investigate the risks of exposure to low dose radiation. The human component of the project focuses on an integrated biology approach using a case-by-case design matched on age, sex and residency, but differing for exposure to radioiodine. Data on genomic copy number, genotype, gene expression, miRNA, Ret rearrangement, pathology and clinical data from these cases are collated in a central source and going to be analysed using an integrative approach in order to achieve highest efficiency and most possible output. One of the biggest hurdles in the correlation analysis of large-scale datasets is the decrease of statistical power due to correction of p-values for multiple testing (Hochberg and Benjamini 1990). P-values by nature are normal distributed and consequently a large number (> 100) of statistical tests due to a high number of data points per dataset is accompanied by false-positive results (type I errors). In studies with smaller numbers of cases this shrinks the statistical power in such an extent that it is almost impossible to detect important

associations. Most often the statistical power of a study cannot be improved by increasing the number of studied cases. An alternative solution to this problem is reduction of data complexity by eliminating those data points of the dataset which cannot be expected to correlate with any of the investigated features. For genomic copy number data this can be done by including only those genomic regions in the analysis which show copy number alterations in a sensible proportion of cases (van de Wiel and van Wieringen 2007). In the described setting in which data from the same samples are available from various platforms even more options of reducing data complexity are possible. Thus further complexity reduction can be achieved by taking only data points into account which correlate with the findings from one or more of the other platforms. This could be genomic copy number data correlating with mRNA or miRNA expression, mRNA expression correlating with SNP patterns or miRNA expression associated with genomic copy number alterations (van de Wiel and van Wieringen 2007; van Wieringen and van de Wiel 2009). Another way of substantially reducing data complexity is looking at genes which participate in a particular pathway only or collections of candidate genes gathered from own or published results. After reduction of data complexity, results are correlated with pathomorphology, molecular biological status such as B-raf mutation status or Ret expression status.

As an extension to this strategy the effect of SNPs on gene expression and miRNA expression will also be examined, as this has already been shown to be important in thyroid cancer (Jazdzewski, et al. 2008). The results are predicted to identify pathways that are important in thyroid carcinogenesis and provide data for future functional studies on novel genes in thyroid cancer. This project offers a new paradigm for cancer research in general, and highlights the future research possibilities of access to large numbers of quality assured biosamples which will become available to the research community as biobanking activity increases.

## 6. Conclusion

It is evident from a large number of studies on thyroid follicular-cell neoplasia that chromosomal aberrations are good starting points for the search of novel cancer genes involved in tumourigenesis. Advanced molecular cytogenetic techniques facilitate this effort. It has been shown in this review that an integrative approach combining data from this genomic analysis with expression and clinical data is straight forward to identify genetic markers that may represent novel therapeutic targets in thyroid cancer.

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## Captions

### **Figure 1:** Work-flow of array comparative genomic hybridisation (array CGH)

A: DNA is isolated from either fresh/fresh-frozen tumour tissue or from formalin-fixed paraffin-embedded tissue and labelled with the fluorescence dye Cy3. Normalized (pooled) DNA from at least five healthy donors is labelled with the fluorescence dye Cy5. B: Labelled tumour and reference DNA are co-hybridised to the array slide in the presence of human cot-1 DNA. Non-specifically bound DNA is washed off the array slide. C: The hybridised array slide is scanned using an array laser-scanner and fluorescence intensities saved in a spreadsheet file. The raw fluorescence ratios (Cy3/Cy5) for each spot on the array are averaged and normalised. The normalised log<sub>2</sub>-transformed ratios are ordered according to the position of clones on the genome and the profile divided into parts sharing the same level of copy number (segmentation e.g. using Circular Binary Segmentation (CBS) by Venkatraman et al., 2006). In the final step the segments are classified as normal, gained and lost (e.g. using the CGHcall algorithm of van de Wiel et al., 2007).

### **Figure 2:** Finemapping of chromosomal breakpoints by FISH using large-insert clones

A: Karyotype of cell line S399 after spectral karyotyping (SKY). Each chromosome is FISH labelled with a chromosome-specific mix of fluorescence dyes (left). The DAPI counterstaining image of chromosomes is transformed into a G-banding like image (middle). The fluorescence image of each chromosome is transformed into a chromosome-specific false colour. Inter-chromosome translocations are identifiable by colour-junctions in the false-colour images (white arrows).

B: Mapping of chromosomal breakpoints of rearranged chromosomes 7 and 12. SKY images of chromosomes 7 and 12 are shown in the top panel. Middle panel: A q-terminal part of chromosome 7 inserted into the q-arm of chromosome 12. The breakpoints were narrowed down using a series of YAC and BAC clones in FISH hybridizations. YAC CEPH-953C9 binds to a region on chromosome 7 (which was inserted into marker chromosome 12) spanning the breakpoint and therefore gives three signals: one on normal chromosome 7, one proximal and one distal the breakpoint on the part of chromosome 7 (indicated by white arrows). Bottom panel: BAC clone RP11 binding

spanning the breakpoint of chromosome 12q resulting in three signals (one on normal chromosome 12, one distal and one proximal the breakpoint).

C: Genomic regions covered by breakpoint mapping FISH clones (UCSC Genome Browser). Upper panel: YAC clone CEPH-953C9 (Chr. 7q) – comprising candidate genes X, Y, Z. Lower panel: BAC clone RP11-1136G11 – comprising candidate genes: Spryd3, Igfbp6, Soat2, Acat2, Csad, Pcap, Znf740, Itgb7, Rarg).

**Figure 3:** Integrative bioinformatic approach as used in the Genrisk-T project

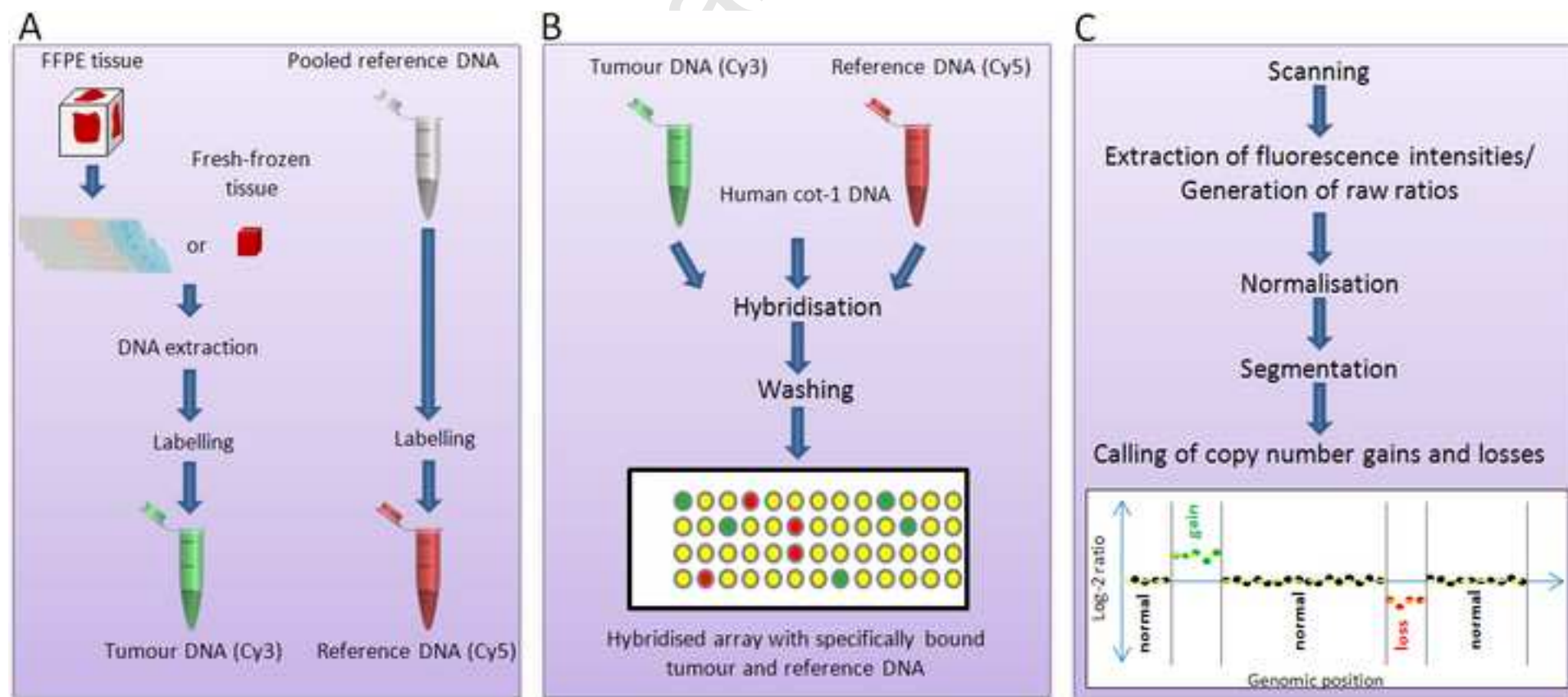
For every sample data from various sources such as array CGH, whole genome SNP genotyping, expression microarray and miRNA array are available. The complexity of each of the datasets is reduced in order to improve statistical power (e.g. taking only copy number regions into account which correlate with mRNA expression, for details see text). The reduced datasets are subsequently analysed for association with pathomorphology, molecular phenotype and clinical information. In case of a statistical significant association molecular profiles either can be directly used as biomarkers or as a starting point for the development of biomarkers.

Table 1: DNA copy number changes in thyroid follicular-cell neoplasia

Tumour type	Number of tumours analysed	Frequent copy number changes <sup>1</sup>	References
PTC	26 tumours	CGH Gains: 1q23-qter, 5q, 7, 17, 21	Hemmer et al., 1999
PTC (classic and follicular variants) and PTC cell lines	8 tumours, 3 cell lines	aCGH Gains: 1, 5, 7, 11, 15, 17, 22 Losses: chromosomes 4, 18, 19	Finn et al., 2007
PTC	60 childhood tumours	CGH Gains: 2, 7q11.2-21, 13q21-22, 21 Losses: chromosomes 16, 20q, 22	Richter et al., 2004
FTC	20 tumours	CGH Gains: 1q, 14q11.2-q22, 17q, 18q12.1-q21 Losses : 1p21-22, 13q21-22, 22	Hemmer et al., 1999
FTC, FTA	12 adenomas, 20 carcinomas	CGH Gains : 5p11, 5p15, 5q13-22, 5q21-23, 7p11-12, 7q11.3-q21, 7q31, 12p11, 12q11-12 Losses : 1p, 2p/q, 3p/q, 6p/q, 8p/q, 9p, 11q, 13q, 15q, 18q, 22q	Roque et al., 2003
FTC, FTA	10 fetal/embryonal adenomas, 4 common adenomas, 4 carcinomas	CGH Gains: 4, 5, 7, 9, 12, 14, 16, 17 Losses: 2, 11, 15q	Castro et al., 2005
ATC	13 tumours	CGH Gains: 7p22-pter, 8q22-qter, 9q34-qter Losses: 22	Hemmer et al., 1999
ATC	9 tumours, 2 cell lines	CGH Gains: 3q, 5p, 7p, 8p, 8q, 11q, 20q Losses : only in single cases	Wilkins et al., 2000

ATC	15 tumours	CGH Gains: 1p34-36, 3p13-14, 5p15, 6p21, 8q23, 9q34, 11q13, 17q25, 19p/q, 20q Losses : 2q32-33, 4q11-13, 5q11-31, 6q21, 8p, 13q21-31	Wreesmann et al., 2002
ATC	10 tumours, 1 cell line	CGH Gains: 1q21-qter, 7p21.3-pter, 20 and CNA in single cases Losses: 3p12.2-21.3 and CNA in single cases only	Miura et al., 2003
ATC	7 tumours	CGH Gains: 3q, 5p, 20p/q and CNA in single cases Losses: Xp, 7q31	Rodrigues et al., 2004
ATC	28 tumours, 9 cell lines	Array CGH Gains: 1q21, 6p22- q21, 7q11.22-q11.23, 11q13, 12q13, 16p11.2, 17q21, 19p13, 19q13.1- q13.2, 20q11.2, 20q13.12, 22q11.21, 22q13.1 Losses :4q12-q13.1, 4q28.3, 13q21.2- q21.31	Lee et al., 2008

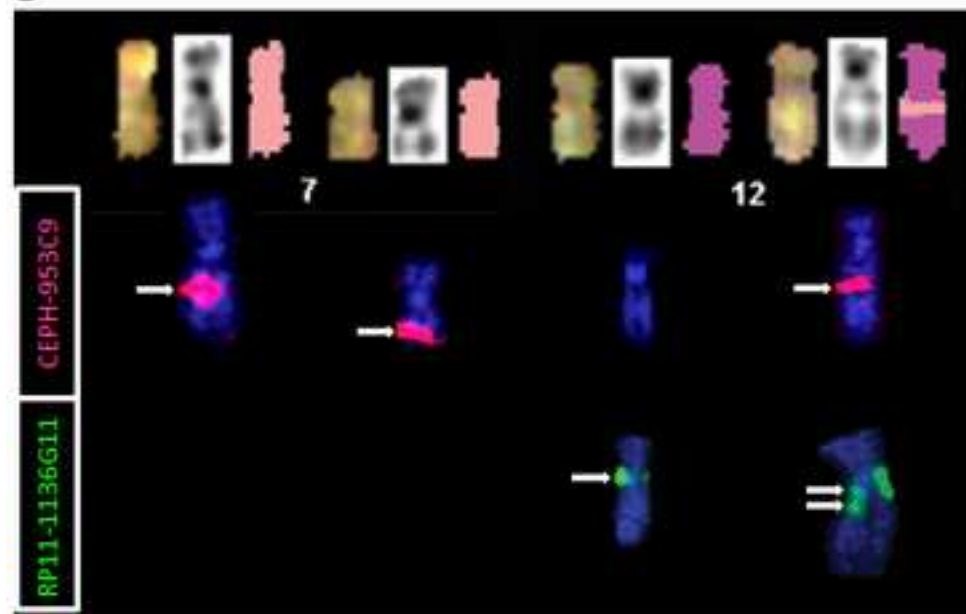
1 : array-CGH (aCGH) or conventional CGH (CGH)



A



B



C



