Chapter 7

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Molecular biology studies of Ukrainian thyroid cancer after Chernobyl

Cancer is known to be an extremely complex disease that is caused by a variety of different agents, including radiation. An understanding of the molecular mechanisms that generate cancer, and the link between molecular features and aetiology, prognosis and response to treatment is pivotal in the successful treatment of the patient. The unprecedented increase in thyroid cancer following the accident at the Chernobyl power plant provided the opportunity to understand the molecular biology of this type of malignancy, and to link molecular features with radiation aetiology. Thyroid cancer in young people is a rare disease, with an incidence rate of around 0.5–1.5 cases per million per year [1]. Initial reports suggested that thyroid cancers that were observed in young people following the Chernobyl accident were different from those from an unexposed population. However, more careful analysis showed that this rare variant, a solid type of papillary cancer that showed extensive invasion, was common in young patients, whether exposed to radiation or not [2]. This contrasts with the classic and follicular variants of papillary carcinoma found more commonly in adults. Since the pathological appearance of a tumor is related to the integration of the molecular events that are involved in its generation, a number of studies have concentrated on defining the various genetic and epigenetic changes involved.

Cases from Ukraine, provided via the Chernobyl Tissue Bank (CTB) have been used extensively in these analyses. The early studies concentrated on the frequency of activation of oncogenes already known to be pathogenic in papillary thyroid carcinoma (PTC). However, more complex molecular techniques have developed in the last decade, and the later studies concentrate on the identification of multiple molecular changes in these tumors.

Changes in the Genome

Somatic DNA – single oncogene studies

Genetic alterations that induce the Mitogen activated protein kinase (MAPK) signalling pathway are common in sporadic PTC. The MAPK pathway is involved in regulation of differentiation, survival and cell growth, and its malfunction is well known to be tumor

promoting [3]. Common alterations include both gene rearrangements and point mutations. Inversions and translocations of a given chromosomal region can lead to an activation of an oncogene that is inactive in the normal thyroid cell. The commonest rearrangements in PTC are those that involve paracentric inversion of part of chromosome 10, *RET/PTC1* and *RET/PTC3*, resulting in fusion with the *H4* gene (*CCDC6*, *D10S170*) and *NCOA4* gene (*RFG*, *ELE1*), respectively. The *BRAF* oncogene which is frequently mutated (point mutation) in adult PTC has also been found to be activated in a small number of radiation-induced PTC following inversion on chromosome 7q [4]. Alterations in the *BRAF* and *RET* oncogenes are usually mutually exclusive [5,6].

Other studies have identified chromosomal breakpoints on 1p32-36, 1p11-13, 1q22, 3p25-26 and 7q32-36 [7-9] and a deletion on 11q [10], but their importance in radiation-induced childhood PTC remains unclear. One reason why some rearrangements occur more frequently than others may be the close proximity of the two gene loci participating in the rearrangement within the nucleus. This theory is supported in a study by Nikiforova *et al*. on the PTC specific translocation of the *RET* and *H4* genes thereby forming *RET/PTC1* [11]. In vitro irradiation studies show increased DNA end-joining enzymatic activity which argues in favour of a radiation-specific response related to gene rearrangement in thyroid cells [12].

RET/PTC gene rearrangement in Ukrainian cases

The *RET* gene is located on chromosome 10q11.2. Its breakpoints for rearrangement are all located within intron 11. The encoded receptor tyrosine kinase has an extracellular domain, a transmembrane domain and an intracellular domain [13]. Fusion of *RET* with other genes provides the tyrosine kinase domain with a new promoter and 5' coding region which leads to constitutive expression of the protein and transmembrane domain is substituted by dimerization domains for ligand-independent activation [14]. There are 17 different *RET/PTC* rearrangements that have been identified in PTC, but inversions of chromosome 10 resulting in *RET/PTC1* and *RET/PTC3* are the most frequent. The *RET* proto-oncogene was already known to be involved in papillary thyroid carcinogenesis in studies of sporadic cases where rearrangements have been found in up to 40% of cases. Rearrangement has been shown to be restricted to the papillary type of thyroid cancer [15,16] and occur frequently in small papillary carcinomas suggesting that it is likely to be a driving event in early tumorigenesis. The breakpoint in the DNA occurs within an intron, which permits the use of reverse transcriptase PCR (RT-PCR) using RNA extracted from the tumors as a useful tool for identifying PTCs that harbour *RET/PTC* rearrangements. However, studies using this technique need to be interpreted with caution, as it has been shown that the results are heavily influenced by the PCR protocol used and tumor heterogeneity [17].

Initial studies post Chernobyl were carried out on Belarussian cohorts [18-20] and suggested an increased frequency of *RET/PTC* rearrangement in radiation induced PTCs in children. In a study on Ukrainian cases, reported by Santoro *et al*. [21], of the 106 papillary carcinomas which produced amplifiable RNA and were analysed for *RET* rearrangement using rearrangement specific RT-PCR, 20 were identified as positive for *RET/PTC1* and 15 for *RET/PTC3*; one other tumor was positive for both *RET/PTC1* and *RET/PTC3*. Analysis of the expression

of the extracellular and tyrosine kinase domains was also performed independently in a different institute in 45 of the 106 cases. Twenty-two of these 45 tumors were positive for expression of *RET* tyrosine kinase. No tumor sample showed positivity for the expression of the *RET* extracellular domain only, suggesting that the *RET* tyrosine kinase expression was due to the presence in these tumors of a *RET* rearrangement fusing the tyrosine kinase domain of the *RET* gene with an active promoter. Among these 45 cases investigated for *RET* tyrosine kinase expression, there were 20 with an identified *PTC1* or *-3* rearrangement, including one tumor which showed both *PTC1* and *-3*; 18 showed *RET* tyrosine kinase expression. The unexpected negative *RET* tyrosine kinase in two cases may have been the result of a lack of sensitivity. Twenty-five cases lacked either a *PTC1* or *-3* rearrangement, 21 were negative for *RET* tyrosine kinase expression, while four were positive. The positive *RET* tyrosine kinase in these four cases could have resulted from the presence of a *RET* rearrangement other than *PTC1* or *-3*. Overall agreement between the two techniques was therefore present in 39 out of 45 cases (87%). The type of *RET* rearrangement correlated with tumor morphology; *RET/PTC3* dominated in the predominantly solid subtype and *RET/PTC1* was more common in the classical subtype of PTC. This is consistent with previous studies on other Chernobyl-related tumor cohorts [20,22,19] and with transgenic models of thyroid cancer in animals [23,24]. A cohort of 28 PTCs from Ukraine and 39 from Belarus was examined by Thomas *et al*. [25]. Patients received surgery 9-11 years after exposure to radiation and their age range at operation was between 6-18 years. 60.7% of the Ukrainian and 51.3% of the Belarussian cohort were *RET/PTC* positive. 23 PTCs harboured the *RET/PTC3* rearrangement and 14 patients the *RET/PTC1*. *RET/PTC3* was found to be associated with the dominantly solid subtype of PTC, whereas *RET/PTC1* was associated with the classic subtype of PTC. Matched normal thyroid tissue samples were negative for *RET* rearrangements. In addition, lymph node metastases of 8 Ukrainian patients were examined. *RET* rearrangement was detected in all cases where the rearrangement was already present in the primary tumor. One metastasis was found to be *RET/PTC* positive although the primary tumor was negative. It had been shown that both ionizing and external radiation [26] were able to induce *RET* rearrangements *in vitro* [27], suggesting that *RET/PTC* rearrangement in these tumors was a potential marker for radiation exposure. *RET/PTC3* rearrangement is more prevalent than *RET/PTC1* and linked to the solid subtype, which was, at the time believed to be more frequent in radiation induced tumors. In the rare cases where it occurs in adult onset sporadic PTC, it was linked to a more aggressive, invasive phenotype with a higher potential to metastasize [28]. Since the early pathological studies suggested that the PTCs found in children post Chernobyl were more aggressive than their adult counterparts, it seemed logical to suggest that this rearrangement was important in the solid subtype, aggressive PTCs, that were frequent in the short latency PTCs, which were being examined at that time.

However, in all the studies reported above there was an inherent problem. Frequencies of *RET* rearrangement in young onset, post Chernobyl PTCs were compared with the frequencies of this oncogene in adult PTCs. Later studies [29], some using age-matched controls [30], have shown that the frequency of *RET* rearrangement in early onset PTC, regardless of prior radiation exposure of the patient, is much higher than that found in adult PTC, suggesting that the initial conclusions on *RET* rearrangement being a biomarker for radiation exposure were incorrect.

Tumor heterogeneity

RT-PCR is a useful tool, but does not allow identification of the proportion of cells that carry the *RET/PTC* rearrangement. It was noted that the strength of RT-PCR signal for *RET/PTC* varied considerably between cases, and that this might suggest that this may represent intratumoral heterogeneity. Unger *et al*. [31] therefore examined 32 patients from the Ukrainian cohort with a tumor latency of 9-12 years for the intratumoral distribution of the *RET/PTC* rearrangement using FISH. FISH is a DNA hybridization method targeting metaphase chromosomes or interphase nuclei on tissue sections. FISH is capable of detecting *RET* rearrangements without further knowledge of the fusion partners. It allows for examination at the single cell level and can contribute to a better understanding of the tumor heterogeneity. Interphase FISH was chosen for the detection of the chromosomal alterations involving the *RET* gene, [31,32]. Two YAC probes including regions within the *RET* target locus and distal to the *RET* sequence were chosen and labelling was performed using two different fluorescent dyes (Fig. 7.1). A confocal laser scanning microscope was used for visualisation. Cell nuclei were scored for the presence of a separated red and green FISH signal in addition to an overlapping signal. This helps to avoid misclassification, which is dependent on the location of the interphase chromosome 10 within the nucleus and the spatial proximity of *RET* to putative rearrangement partners. Overlapping signals occur only in normal nuclei, and split signals indicate an aberration of the *RET* gene. This approach has been further validated by using careful selection of controls, including *RET/PTC* positive and negative cell lines as well as wild type *RET* expressing controls (Fig. 7.2).

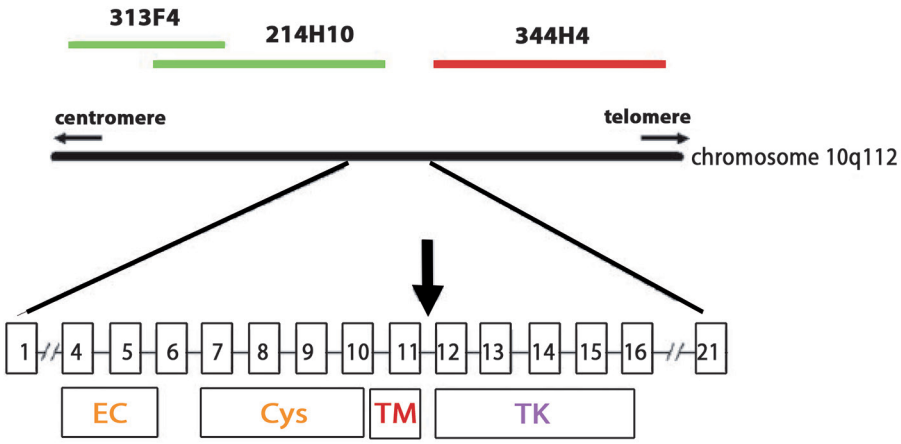

Figure 7.1. Mapping of YAC probes 313F4, 210H10 and 344H4 on chromosome 10q11.2. YAC clones 313F4 and 214H10 (FITC-labelled, green) map proximal to and include the *RET* locus, 344H4 (Cy3-labelled, red) maps distal to *RET*. Exons and different parts of the RET gene are indicated below (EC=extracellular domain, Cys=cystein-rich domain, TM=transmembrane domain, TK=tyrosine kinase domain).

The study reported by Unger *et al*. [31], comprised 29 PTCs, two follicular adenomas, and a follicular cancer. Detection of the *RET* rearrangement with conventional qRT-PCR revealed that 13 (40.6%) of the patients expressed the TK domain of *RET*, and in only

two and three patients, respectively, was a clear *RET/PTC1* and *RET/PTC3* signal detected. Investigation of the cases with FISH was more sensitive and 23 patients (72%) were positive for the *RET* rearrangement. A threshold for positivity was set as evidence of rearrangement in a minimum of 7% of cells within a tumor. The highest observed frequency of cells bearing the *RET* rearrangement positive FISH signal was 46%. In none of the tumors was a FISH positive signal observed in 100% of tumor cells. Further analysis showed that the distribution of FISH positive cells was inhomogeneous.

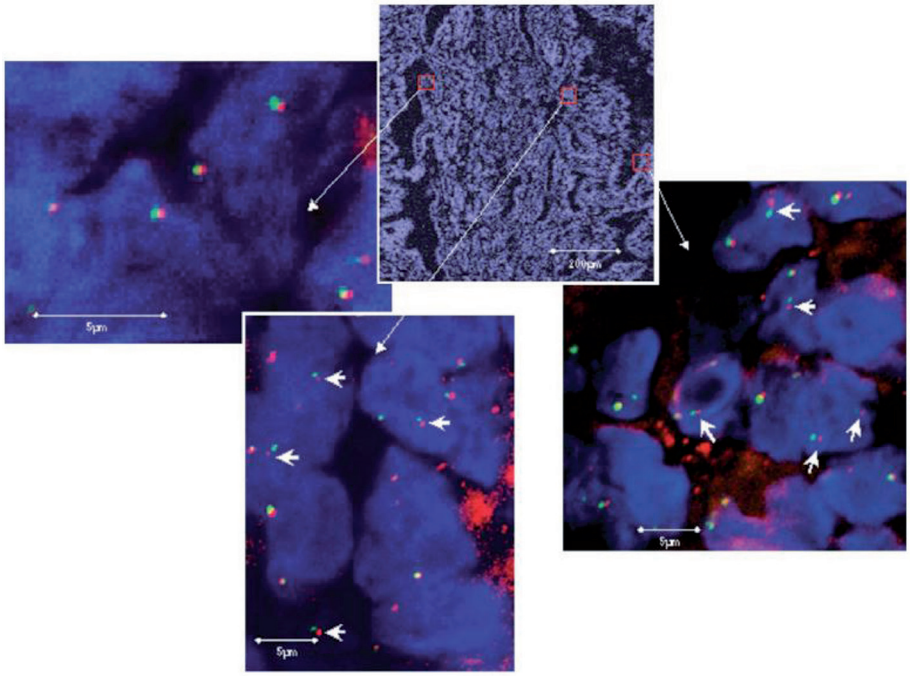

Figure 7.2. Example of FISH analysis with *RET*-specific YAC probes using confocal laser scanning microscopy (CLSM). Three different areas were randomly selected from the tumor as indicated at the 40x magnification image (TOPRO image only, top center). One viewing area (left side) shows only normal cells exhibiting overlapping FISH signals. Two other viewing areas (middle and right side) show a cluster of aberrant cells identified by split FISH signals (arrows). All images are superimposed from approximately ten different slices throughout the thickness of the tissue section. For a more precise evaluation a stepwise scoring every 0.5 µm is applied.

The same group repeated the *RET* rearrangement analysis on a Ukrainian cohort of 13 patients presenting with PTC after a shorter latency of 4-8 years post Chernobyl [32]. They compared this short latency group to the long latency group described previously for the distribution of the *RET* rearrangement within a given tumor sample. Examination with interphase FISH showed 10 of the patients (77%) were positive for a *RET* rearrangement with a frequency of rearranged cells between 11-54%. In 9 cases, the *RET* rearrangement was homogenously distributed all over the tumor tissue, whereas one case showed distinct clustering in certain areas of the tumor. Both studies identified a similar frequency of intertumoral rearrangement of *RET*, but the intratumoral distribution of the

RET rearrangement between the groups differed significantly. The longer latency group included a higher frequency of tumors with a heterogenous distribution and clustering of the rearrangement. At the same time, others had noted that tumor morphology was changing from a predominantly solid subtype in the shorter latency to a solid-follicular subtype in the longer latency group [33]. The reason for this may be the outgrowth of distinct subclones in longer latency tumors or the presentation of *RET/PTC* as a second event in thyroid carcinogenesis.

In a later study, reported by Hieber *et al*. [34], interphase FISH analysis was used to assess *RET* rearrangements of the cases. Aside from five other chromosomal rearrangements, 16 of the cases (72%) were positive for *RET* rearrangements. This is a rate similar to that observed in other studies [31,32]. With a threshold for *RET* positive of 7.1%, the number of rearranged cells per case varied from 10.6% to 41.5%. There was no obvious correlation with age or gender of the patients, but the median age at operation of the patients carrying rearrangements was higher (22 years) compared to the group with no rearrangement (18 years). Within the group carrying rearrangements, older patients showed greater tumor heterogeneity with respect to *RET/PTC* rearrangements, consistent with the earlier study by Unger *et al*. [31].

The BRAF proto-oncogene

BRAF is a RAF kinase which plays an important role in the MAPK pathway. It is member of the family of RAF proteins, and due to its serine/threonine kinase activity it phosphorylates the MAPK/ERK kinase upon stimulation by RAS [35]. It is frequently mutated in thyroid cancer, where the point mutation at nucleotide position 1799 is the most prominent one. A transversion from thymine to adenine (*T1799A*) changes the amino acid sequence in codon 600 from valine to glutamic acid (*V600E*); thus the abbreviations used to designate this mutation are $BRAF^{T1799A}$ or $BRAF^{V600E}$. This results in constituent activation of the gene, thus activating the MAPK pathway. The frequency of mutation varies in a number of studies from 36-69% in adult papillary thyroid carcinoma [36,37], including one study on Ukrainian tumors [30]. In adult cases, *BRAF* mutation significantly correlates with clinical parameters in PTC such as distant metastases, advanced clinical stage and disease specific mortality [38]. In children, the involvement of *BRAF* has been found to be somewhat different. Kumagai *et al*. [39] performed a study on childhood PTC from Japan and Ukrainian patients with PTC following the Chernobyl accident, and compared the frequency of *BRAF* mutation with that found in adult PTCs. Sequence analysis of the $BRAF^{T1799A}$ mutation in exon 15 found only one positive case in the young Japanese cohort. No mutations in *K-, H- or N-RAS* were detected. The same result was obtained for the childhood group (aged under 15 at diagnosis) within the Ukrainian cohort. However, among the cases of young adults, 8 out of 33 carried the $BRAF^{T1799A}$ mutation and two other cases carried *RAS* mutations, one in *NRAS* codon 61 and one in *HRAS* codon 61. The Ukrainian group was also examined for *RET* rearrangements. 5 out of 15 in the childhood group exhibited the rearrangement, and 12 out of 33 in the group of young adults. Mutation of *BRAF* and rearrangement of the *RET* oncogene were mutually exclusive. Interestingly, in this study, the presence of the mutation was not

associated with large tumor size, regional lymph node invasion, extrathyroidal invasion, or distant metastases.

Lima *et al*. [40] examined a cohort of 34 post-Chernobyl paediatric PTC from Ukraine and found that four were positive for the *BRAF*^{V600E} mutation. 14 of the patients had a *RET/PTC* rearrangement, but *BRAF* mutation and *RET* rearrangement were, again, mutually exclusive. The *BRAF* mutation occurs predominantly in the papillary and follicular variants, whereas the *RET* rearrangements were more common in the solid subtype of the tumor. The control group of sporadic pediatric PTCs contained no solid variants, and the frequency of *RET* rearrangement was not determined. Powell *et al*. [30] used 67 cases of post-Chernobyl PTC from Ukraine to examine whether the *BRAF* mutation was related to the age of the patients or the radiation exposure. 32 patients were aged above 30 at time of clinical presentation, and 35 patients were under 16. All patients, exposed or unexposed to radiation, were residents in the Ukraine and hence an ethnic difference as confounder can be excluded. Paired tumor – normal samples were analysed and 18 out of the 32 adult cases were found to harbour the *BRAF*^{V600E} mutation in the tumor but not in the normal tissue. The tumor morphology was of dominantly classic or dominantly follicular subtype. Only one child in the group of 35 exposed cases was positive for the mutation and none of the sporadic PTCs. Interestingly, the one case bearing the *BRAF* mutation was of a dominant papillary subtype.

In summary, *BRAF* mutations are more commonly seen in PTCs with a dominantly papillary morphology, and are more common in PTCs diagnosed in adulthood rather than childhood. The low frequency of *BRAF* mutation in PTCs from children exposed or not exposed to radiation suggests that, as with *RET* oncogene rearrangements, the age of the patient at diagnosis is a significant factor. It is therefore important to ensure that cases are appropriately age matched when seeking to identify radiation related changes in molecular biology of tumors. One study has also shown that in post-Chernobyl PTCs from Belarus, *BRAF* can be activated by gene rearrangement. In this case, the C-terminal region of the *BRAF* gene is fused to the first eight exons of the *AKAP9* gene building the *AKAP9/BRAF* fusion gene [4]. However, as is the case with the early studies of *RET* rearrangement in post Chernobyl PTCs, this study lacked age matched controls and it is therefore unclear whether this finding is related to radiation or to the age of the patient at operation.

Other proto-oncogenes

Although *RET/PTC* and *BRAF* alterations are the commonest oncogene alterations seen in PTC, rearrangements and mutations of other genes are also observed, but at a much lower frequency. *NTRK1* encodes the high affinity nerve growth factor (NGF) receptor, and is occasionally rearranged in PTC. *NTRK1* fusion partners are the genes *TMP3*, *TPR* and *TFG* [41,42]. The *NTRK1* rearrangement, is only present in about 11% of sporadic PTCs [43], and is rare (3.3%) in post-Chernobyl thyroid tumors [44-46].

Other types of thyroid cancer are known to involve much different oncogenes, such as the *RAS* oncogene [47] in follicular carcinoma or *TP53* in anaplastic carcinoma [48]. Mutations of the thyroid stimulating hormone receptor (*TSH*R) gene occur in follicular carcinoma and benign thyroid tumors [49,50]. Santoro *et al*. [21] examined whether one

of these molecular changes had an influence on the development of PTC after exposure to radiation. The group studied 128 PTC of post-Chernobyl patients who received surgery before the age of 15, all of whom were from Ukraine. In addition to *RET* rearrangement, reviewed in the section above, mutations in the *RAS, TP53* genes, and exon 10 of the *TSHR* were also analysed and correlated with the morphological status of the tumor samples.

Alterations in the other genes of interest were not found to be present in the post-Chernobyl PTC cohort. Other studies could also not demonstrate correlation of *TSHR* or *TP53* mutations in thyroid carcinogenesis after radiation exposure [51-54]. *RAS* mutations are not commonly found in sporadic PTCs, although they are identified in follicular thyroid tumors, and detection in radiation-induced childhood PTC is similarly rare [39,53,55].

These results show that mutations in the *TP53* and *H-, N-*, and *K-RAS* genes play no important role in the carcinogenesis of radiation-induced PTC.

Studies on genomic profiling

Array-based comparative genomic hybridisation (aCGH) is a technique that allows an objective and quantitative examination of copy number changes of the entire genome at a high resolution. BAC clones covering the whole genome in 1MB distances are spotted on glass slides and used for hybridisation with probe sequences of a tumor sample. Genomic alterations such as deletions or amplifications are detected with a greater sensitivity and improved resolution (1MB) than by karyotyping, but chromosomal rearrangements such as translocations and inversions cannot be identified using this technique. aCGH is also capable of detecting copy number variations which occur regularly in normal tissue and need to be distinguished from their pathogenic counterparts.

Unger *et al*. [56] analysed 33 patients who developed PTC following the Chernobyl accident for chromosomal aberrations using aCGH and validated the results by FISH. The cohort was a mix of 13 children and 20 adults with known *RET/PTC* rearrangement status. *RET/PTC* positive cases and *RET/PTC* negative cases were compared, as well as children versus adults. In all cases, deletions occurred with a higher frequency than amplifications. This might have been predicted, as radiation is thought to introduce chromosomal breaks which may lead to deletions, translocations or inversions rather than a chromosomal gain. Losses were predominantly on chromosomes 1, 6, 7, 9, 10, 11, 12, 13, 16, 19, 20, 22 and gains on chromosomes 10, 12, 19, 20, 21. Hierarchical clustering distinguished the three groups; childhood *RET/PTC* positive, adult *RET/PTC* positive and adult *RET/PTC* negative. *RET/PTC* positive cases differed significantly for a region on chromosome 1p which was deleted in adults, but not in children. All *RET/PTC* positive cases differed significantly from the negative cases in losses on 1p, 7p, 9, 13q and gains on 3q, 4p, 12q, 21q. Deletions of regions on 9q, 10p, 10q and 22q had also been observed in other studies on thyroid tumors [57-61]. Hence, these aberrations seem to be more specific for carcinogenesis in thyroid than for the exposure to radiation and hence cannot be considered as radiation specific biomarkers. Gene detection in the altered regions revealed 31 candidate genes which are known to be involved in tumor progression and 21 tumor suppressor genes which specifically map to deleted chromosomal regions. It is of note that the majority of these genes is known to be involved

in thyroid carcinogenesis, and a further study giving a comparison with sporadic PTCs would be necessary to define a specific role in radiation induced tumor development. The genes identified are involved in the molecular pathways of apoptosis, interleukin-27, angiopoietin receptor and the PI3K/MAP kinase. The data reflects a large heterogeneity in post-Chernobyl PTC which suggests the existence of various routes of tumor development. In terms of biomarker screening this may imply that a whole panel of markers will be necessary for an exact detection of a radiation signature.

Stein *et al*. [62] used tumor and matching normal tissue of 10 Ukrainian patients with post Chernobyl PTC to examine copy number changes and gene expression. The latency for these tumor patients was 13-14 years, and age at exposure varied from 3 months to 18 years whereas age at operation was from 14-31 years.

In studies on sporadic PTCs, copy number alterations (CNAs) are detected with a low frequency. Depending on the study, the occurrence of gains and losses ranges between 2-50% [7,59,63]. Detection is strongly dependent on the method used and the conventional CGH and cytogenetic approaches used in these studies have not the same high resolution as the currently used array-based CGH (aCGH). Stein *et al*. used high resolution SNP array CGH technology for CNA examination. Several amplifications on chromosome 22 were detected in all 10 samples. Also, chromosomes 1 and 12q show amplifications with high frequency. Further, less frequent amplifications on 5p, 9q, 16p, and 21q were also detected. Deletions were identified in chromosomes 21q and 14q, and several deletions on 1q, 6p, 9p, 10p, 13q, and 22 were restricted to 2 tumors specifically. In general, deletions were less common in PTC than amplifications independently from radiation exposure or sporadic occurrence. Although radiation is thought to generate an increased amount of deletions, translocations and inversions due to chromosomal damage, there is no direct evidence that the number of chromosomal aberrations in radiation-induced PTC is higher than in sporadic PTC. However, some aberrations seem to occur preferably after radiation exposure. Chromosome 22 was reported in several studies to be affected in post Chernobyl patients [59,64,65] and was associated with tumor aggressiveness in younger patients. Gene expression of genes in these regions was determined and compared with gene expression in sporadic tumors. 41 genes were specifically upregulated in post Chernobyl pediatric PTC but not in sporadic PTC, and the ones with the strongest overexpression were *TESC*, *PDZRN4*, *TRAA/TRDA*, *GABBR2*, *CA12*, *MPZL2*, *SCG5*, *PDZK1IPI*, *AMIG02*, *NOVA2*, and *TNIK*. The genes with the largest downregulation of expression from a total of 24 genes were *PAPSS2*, *PDLIM3*, *BEXI*, *ANK2*, *SORBS2*, *PPARGCIA*, *MT1M*, *CTGRF*, *LYVE1*, and *OGDHL*. Only one gene on chromosome 22, *FBLN1*, was reported to be specifically downregulated. It would be of interest to examine these genes further for their potential usefulness as biomarkers of radiation exposure.

Another study on chromosomal aberrations was carried out by Hieber *et al*. [34]. A cohort of 23 patients from the Ukraine who developed PTC after the Chernobyl accident were examined for chromosomal aberrations and *RET/PTC* rearrangements. The median age of the patients at operation was 21 years old. SKY (spectral karyotyping) was performed and revealed chromosomal aberrations in 14 of the cases, most frequent on chromosomes 7, 10, 11, 21, and 22. It is unknown whether these aberrations are related to radiation exposure as no group of sporadic PTC was included in this study [6,13,14]. As discussed earlier, studies on sporadic thyroid cancer had already described aberrations of chromosome 22 as a cytogenetic event in subtypes of PTC.

None of the above mentioned studies was able to define a biomarker specifically targeted to radiation exposure. This may be partially due to the design of the studies as confounders such as age at operation, ethnic origin and histology of the tumor play a role in the molecular phenotype of tumors, and the cohorts studied often lack matched controls.

Hess *et al*. [66] designed a study to specifically address the question whether there are genomic alterations that correlate with exposure to radiation. A cohort of 56 patients with PTC was matched by age at operation and residency. The patients were known to be exposed to radiation (33 patients born before April 1986) or not exposed to radiation (19 patients born after January 1987). A second cohort of 28 PTCs (16 exposed and 12 unexposed) matched on the same criteria was selected to validate the results. aCGH and FISH were performed as reported in a previous study [56] to evaluate chromosomal aberrations that differed between exposed and unexposed cases. Amplifications were found to be more frequent than deletions which are in agreement with the findings of Stein *et al*. [62]. In general, DNA gains (n=81) were more frequent than DNA losses (n=63). Five regions on chromosomes 1, 3, 4, and 12 were lost, and six regions on chromosomes 12, 19, 20, and 22 were gained in all cases investigated. Hierarchical cluster analysis separated the array CGH profiles into two main clusters, 1(n=23) and 2 (n=29). Cluster 2 was subdivided into two subclusters, 2-1 (n=14) and 2-2 (n=15). Cluster 1 contained significantly more *RET/PTC* positive cases compared with cluster 2 (p=0.0096). Large tumors (pT2 and pT3) were associated with cluster 1 (p=0.00087). Tumors that had metastases to regional lymph nodes (N1) were predominant in cluster 1 and cluster 2-2 (p=0.04). Univariate supervised analysis showed associations [false discovery rate (FDR) <0.05] of CNAs with tumor size (gain of 1q21.1–23.3, 7q22.1, 9p24.3, 10p15.3–15.1, 10q26.13–26.3, 11p11.12-cen, 12q24.11–24.23, and 16q22.1–23.3) and sex of patients (loss of 5q23.3–31.3). A DNA gain on chromosome 7 (7p14.1-q11.23, 32.1Mb in size) was exclusively associated with a subgroup of patients exposed to radiation after Chernobyl (univariate supervised analysis; FDR<0.05). The alteration was present in 13 out of 33 cases from the exposed group and in none of the cases from the unexposed group (p=0.0015, FDR=0.035). This finding was verified by array CGH analysis of an independent validation set consisting of 28 PTC cases (16 exposed and 12 unexposed). FISH analysis of individual cases revealed that up to 24% of tumor cells exhibit the amplification of 7q11. As the cohort was matched on the confounding parameters age, residency and *RET/PTC* status, the gain of 7q11 could be described as the first potential marker identified for exposure to radiation. Nine genes identified within this region were associated with tumor development according to gene ontology analysis. The five genes *CLDN3, CLDN4, LIMK1, PMS2L2*, and *RFC2* showed no significant change in expression, whereas the three genes *PMS2L3*, *PMS2L11*, and *STAG3L3* were significantly overexpressed in tumors with gain compared to those with normal copy number. *CLIP2* was found to be overexpressed in exposed tumors in general, independently from the presence of the gain of 7q11. *CLIP2* (Cap-Gly domain main containing linker protein 2) is a relatively unknown gene with putative functions in microtubule formation, chromosome segregation and cell division. As these are mechanisms which become perturbed in cancer development, there might be a cancer-related function of *CLIP2*, but its involvement in tumorigenesis, and especially radiation-induced tumor development, is currently unknown.

Interestingly, the gain on chromosome 7q11 was already reported in a previous study of Richter *et al*. [61], who used conventional CGH to analyse chromosomal aberrations in post-Chernobyl PTC. A cohort of 60 children presenting with PTC after the Chernobyl accident was used for the study. The 48 female and 12 male patients were matched on age at exposure, age at operation and residency. 30% (18 cases) had an altered chromosomal profile. The most frequent deletions were observed on chromosomes 16p, 16q, 20q and 22q, and amplifications of chromosomal regions on chromosomes 4, 7q11.2-21, 13q21-22, and 21. The region 7q11.2-21 overlaps with the region described in Hess *et al*., but none of the other CNAs identified in this study correspond with those found by Hess *et al*. The other CNAs idenified may be more related to the age of the patients at operation or the behavioural characteristics of the tumor. Deletions on chromosome 22 occur in PTC of patients of younger age and thyroid carcinoma with an increased invasive and metastatic potential [67,68,59]. Imbalances on chromosome 16 were reported earlier to be involved in various forms of thyroid cancer [69,57].

The results of these studies are summarised in Table 7.1.

Table 7.1
Overview of genomic abberrations found in the Ukrainian cohort

Author [ref.]	chromosomal alterations gains	chromosomal alterations losses	Observed in sporadic cases	genes/pathways
Unger *et al*., 2008 [56]	chromosomes 10, 12, 19, 20, 21	chromosomes 1, 6, 7, 9, 10, 11, 12, 13, 16, 19, 20, 22	chromosomes 9q, 10p, 10q and 22q	apoptosis, interleukin-27, angiopoietin receptor and the PI3K/MAP kinase pathways
Stein *et al*., 2011 [62]	chromosomes 22, 1, 12q, 5p, 9q, 16p, 21q	chromosomes 21q, 14q, 1q, 6p, 9p, 10p, 13q, 22		Upregulated: *TESC*, *PDZRN4*, *TRAA/TRDA*, *GABBR2*, *CA12*, *MPZL2*, *SCG5*, *PDZK1IPI*, *AMIG02*, *NOVA2*, *TNIK*. Downregulated: *PAPSS2*, *PDLIM3*, *BEXI*, *ANK2*, *SORBS2*, *PPARGCIA*, *MT1M*, *CTGRF*, *LYVE1*, *OGDHL*, *FBLN1*
Hieber *et al*., 2011 [34]	chromosome 7, 10, 11, 21 and 22			
Hess *et al*., 2011 [66]	chromosomes 12, 19, 20, 22, 7q11	chromosomes 1, 3, 4, and 12	chromosomes 1, 3, 4, 12, 19, 20, 22	Upregulated: *PMS2L3*, *PMS2L11*, *STAG3L3*, *CLIP2*
Richter *et al*., 2004 [61]	chromosomes 4, 7q11.2-21, 13q21-22, 21	chromosomes 16p, 16q, 20q, 22q	chromosomes 16, 22	

Germline Genome

Genetic association studies using germline DNA variants

Genetic association studies are aimed at the exploration of the link between genotype and phenotype to facilitate the determination of genetic risk factors for the potential development of disease. These studies evaluate gene polymorphism, usually the single nucleotide polymorphism (SNP) in retrospective or prospective case-control studies. In general, there are two methodological approaches to selecting which and/or how many SNPs need to be analyzed. The first one, the so-called candidate gene approach, hypothesizes that genetic variations in one or in a limited number of genes may influence the risk for or the phenotype of a given disease. A more comprehensive way is hypothesis-free; it employs analysis of the whole genome, hence the genome-wide association study (GWAS). To date, a number of investigations have been done in the area of thyroid cancer, mostly in sporadic cases, with a few of those exploring the radiation-induced thyroid cases.

Candidate gene approach

In a study investigating loss of heterozygosity (LOH) for three different SNPs of the *RET* gene (G691S, L769L, and S904S), no evidence of such was observed in 28 of 46 radiation-related papillary thyroid carcinomas (PTC) from Ukraine and Belarus that were heterozygous for at least one of the three SNPs under analysis [70]. A microarray investigation performed later and independently was consistent with these findings [71]. In the additional 68 cases, the rare S allele of G691S was significantly more frequent in patients older than 30 years old as compared to the younger subjects. Since excess radiation risk for PTC in the individuals exposed after they are 20 years old is low and it further declines with age at exposure, the study concluded that *RET* polymorphisms may rather influence sporadic but not radiation-induced thyroid carcinogenesis.

A study of the Arg72Pro polymorphism of the *TP53* gene (which encodes a tumor suppressor protein TP53 commonly reffered to as p53) in 48 pediatric/adolescent and 68 adult patients with PTC exposed to Chernobyl radiation from Ukraine and Russia, and 53 adult patients with sporadic PTC and 313 healthy controls from Russia found that the Arg/Arg homozygotes were significantly less frequent in adult patients than in children and adolescents [72]. No LOH or imbalanced *TP53* allele expression was observed in tumor tissues of heterozygous individuals. It was proposed that *TP53* genotype other than Arg/Arg may contribute to the risk of development of PTC in individuals exposed to radiation during their late childhood, adolescence or in young adulthood, particularly in females aged between 18 and 30. Interestingly enough, an elevated risk for thyroid cancer was reported in females exposed to Chernobyl fallout at the age below 30 years in a radiation epidemiological investigation [73].

A subsequent study extended to the analysis of 9 SNPs in 5 genes involved in DNA damage response (*ATM*, *XRCC1*, *TP53*, *XRCC3* and *MTF1*) performed in 255 PTC patients (123 from Chernobyl areas and 132 sporadic) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure) demonstrated that

the *ATM* G5557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, were associated with a decreased risk of thyroid cancer [74]. Of note, the *ATM* IVS22-77 T>C and *TP53* Arg72Pro SNPs interacted with radiation exposure: the *ATM* IVS22-77 associated with the increased risk of sporadic PTC whereas *TP53* Arg72Pro correlated with the higher risk of radiation-induced PTC in adult patients, in support to the study which involved the Ukrainian patients described above [72]. An issue of gene-gene and gene-environment interactions was addressed in the analysis of *ATM*/*TP53* genotypes. Some of those strongly associated with either sporadic or radiation-induced cancer suggesting that polymorphism of these genes may modify the risk for PTC of different etiology.

Genome-wide association studies

The genome-wide association studies (GWAS), sometimes also referred to as molecular epidemiology investigations, are conducted using advanced technologies to rapidly and cost-effectively analyze genetic differences between individuals with specific diseases compared to healthy individuals. Usually, the studies are performed in two or more stages. First, genotyping of a large number of SNPs in a relatively limited sample set, which is called the training set, is performed on microarrays. The purpose of this step is to tentatively determine the potential candidate polymorphisms at liberal *p* value. Subsequently, genotyping of full or narrower set of SNPs in, again, a relatively small sample set is undertaken to achieve more stringent *p* value. Further on, a validation study is performed by genotyping a narrow set of pre-determined SNPs in an extended sample set to increase *p* value stringency or to disprove the association. This last step is commonly performed in the single-track assays such as e.g. TaqMan. Finally, a strict statistical meta-analysis is done, which combines the results obtained at different stages, with correction for multiple testing. Eventually, one or several SNPs might be determined as conferring the association with the risk for disease. A functional study might be further undertaken to clarify the mechanism by which this SNP or a gene with which this SNP is in strong linkage disequilibrium (LD) might be involved in the pathogenesis of a given disorder.

Before discussing the results obtained in Chernobyl PTCs, it is necessary to mention several appropriately powered studies of genetic predisposition to sporadic thyroid cancer published recently. The first one, employing a sample set of 962 cases of differentiated thyroid cancer (i.e., mostly PTC and limited number of FTC combined) and 38,923 controls, reported rs965513, a SNP located at chromosome 9q22.33, 59 Kb upstream (centromeric) to the *FOXE1* gene encoding a thyroid-specific transcription factor TTF2, as the strongest genetic marker (OR=1.75; 95% CI 1.59-1.94; $p=1.7\times10^{-27}$) associating with thyroid malignancy in individuals of Iceland, Spain and the USA of European descent [75]. This study also claimed another SNP, rs944289, on chromosome 14q13.3 in the area of the *NKX2-1* gene that encodes the TTF1 transcription factor, to be a marker for thyroid cancer (OR=1.37; 95% CI 1.24-1.52; $p=2.0\times10^{-9}$). A consequent study of the same group, based on a sample set of up to 1,150 cases and 41,448 controls, found 3 additional SNP associating with low TSH levels in population also to be the markers of risk for thyroid cancer. These are rs966423 on 2q35 (OR=1.34; 95% CI 1.22-1.47; $p=1.3\times10^{-9}$), rs2439302 on 8p12 (OR=1.36; 95% CI 1.23-1.50; $p=2.0\times10^{-9}$) and rs116909374 on 14q13.3 (OR=2.09;

95% CI 1.68-2.60; p=4.6x10^{-11}) [76]. With regard to genetic markers in the *FOXE1* proximity, it is important to take into account the results of an independent study of 768 tag-SNPs in 97 genes which were initially genotyped in 615 PTC cases and 525 controls from Spain and then in 482 patients with PTC and 532 controls from Italy for validation [77]. The target genes were selected based on their differential expression in primary thyroid tumors or the involvement in thyrocyte biology, metabolism and/or carcinogenesis such as the MAP kinase, JAK/STAT and TGF-beta pathways. An SNP, rs1867277, within the LD block spanning *FOXE1* and located at the 5'UTR of the gene was identified to associate with PTC (OR=1.49; 95% CI 1.30–1.70; p=5.9x10^{-9}). Functional study demonstrated that this SNP affects *FOXE1* expression by differentially recruiting the USF1/USF2 transcription factors.

At present, the results of only one investigation of Chernobyl PTCs from Belarus employing GWAS have been reported [78]. The developments of this project, however, required the involvement of additional PTC cases and controls from Ukraine. Therefore, it seems pertinent to describe this study here.

During the first phase of the molecular epidemiology study undertaken in the frame of Nagasaki University's Global COE Program supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for 2007-12, a total of 667 patients from Belarus diagnosed for PTC in 1989–2009 and 1,275 controls from Belarus and Russia were studied, of which 408 cases and 627 controls were genotyped using the Illumina microarrays accommodating over 500,000 human SNPs; the rest of samples were subjected to the validation study. Statistical meta-analysis has discovered 3 SNPs at chromosome 9q22.33 significantly associating with Chernobyl PTC with a p-value order of 10^{-9} (Fig. 7.3). This value indicated a very significant association which surmounted the genome-wide threshold of 2x10^{-7}. For one of the detected SNPs, rs965513, which was used for validation, a p-value of 4.8x10^{-12} and an odds ratio of 1.65 (95% CI 1.43–1.91) was obtained after meta-analysis.

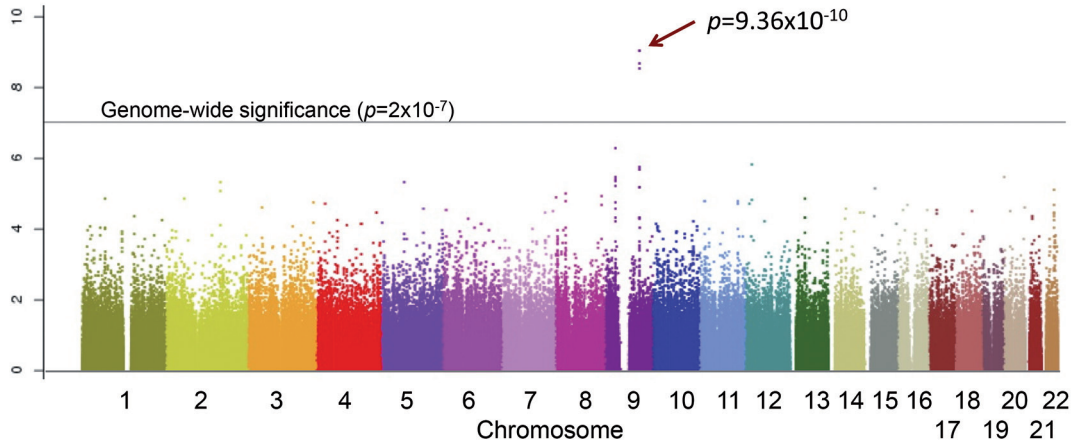

Figure 7.3. Manhattan plot of the GWAS results of the first stage of Chernobyl PTC study. The p values calculated by the Trend chi-square test for 506,840 autosomal SNPs are plotted in -log$_{10}$ scale with respect to their chromosomal positions. The horizontal line indicates the Bonferroni adjusted p value for genome-wide significance.

In the second phase of the study, the sample set was expanded to include additionally 286 PTC cases from Belarus and 145 from Ukraine, and 257 controls from Belarus, 157 from Ukraine and 620 from Poland. Thus, a total of 1,098 PTCs from Belarus and Ukraine, and 2,309 controls (both cases and controls that passed the quality control) from the three countries were included. The number of samples genotyped using Illumina microarrays was 847 PTC cases and 1,240 controls; validation study using single-track TaqMan assays included 274 cases and 1,023 controls.

Among all the targets tested, only 3 SNPs displayed significance in the training and validation sets while all other did not pass validation. Two SNPs were around or in the *FOXE1* gene locus on chromosome 9q22.33: rs965513 (OR=1.69; 95% CI 1.51–1.90; p=5.8x10^{-19}), and rs1867277 (OR=1.52; 95% CI 1.26–1.83; p=1.4x10^{-5}). The third SNP that displayed significant association was in the *NRG1* locus on chromosome 8p12, rs2439302 (OR=1.35; 95% CI 1.16–1.57; p=9.1x10^{-5}). Notably, all three SNPs detected in our Chernobyl series had effect size, in terms of OR, very similar to those reported in sporadic thyroid cancer studies. Based on these observations it was concluded that these SNPs are the markers of PTC of either radiation or sporadic etiology.

It is worth mentioning that besides the 3 SNPs that displayed significant association signals, the study also identified 11 potential candidate SNPs in or around 10 genes which were significant on microarray analysis with p values ranging 4.7x10^{-7} – 1.6x10^{-4}. However, none of them, when subjected to validation study, passed it displaying the p-values from 0.07 – 0.944. Therefore, all of them were conservatively judged as having no association with Chernobyl PTC even though their significance (a p-value) for association ranged 6.0x10^{-6}–5.0x10^{-3} on final meta-analysis. The SNPs in vicinity of *NKX2-1*, *MBIP* and *DIRC3* previously reported to be associating with sporadic thyroid cancer were insignificant in the study thus pointing on the specific association of these genes with sporadic thyroid cancer.

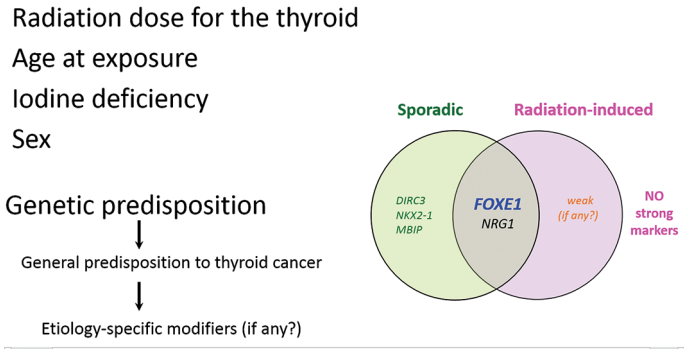

Figure 7.4. Risk factors for development of papillary thyroid carcinoma after internal exposure to radioiodine. Genetic predisposition is added to those previously established to affect the risk: exposure dose, younger age at exposure, iodine deficiency, male sex. Note that genetic markers associating with risk for Chernobyl PTC are the same found for sporadic PTC, around or in the *FOXE1* (the strongest) and *NRG1* gene loci. Several markers appear to be associating with the risk for sporadic thyroid cancer only: in or around the *DIRC3*, *NKX2-1* or *MBIP* genes.

As a whole, molecular epidemiology study of Belarussian and Ukrainian PTCs leads to an important corollary that among the genetic factors affecting risk for radiation-induced thyroid cancer, the strongest markers are the same that confer predisposition to the sporadic form of this type of malignancy. Genetic markers of radiation-related and sporadic partly overlap yet the absence PTC-associating markers on 14q13.3 (i.e., *NKX2-1* or *MBIP*), and 2q35 (*DIRC3*) from the Chernobyl series suggests they are specific to PTC developing without radiation history. As shown in Figure 7.4, the results obtained in the study of Chernobyl PTC permit the inclusion of genetic predisposition to the list of risk factors for radiation-induced thyroid carcinogenesis known from the earlier experience. The fact that GWAS has not revealed genetic markers specifically associating with radiation-related but not sporadic thyroid cancer does not rule out the possibility of their existence. Further studies with higher resolution, such as e.g. next-generation sequencing, may shed light on this problem and will probably refine our understanding of radiation-induced carcinogenesis by addressing issues of gene-gene and gene-environment interactions.

Possible implication of FOXE1 in pathogenesis of PTC

The three genetic studies, two of sporadic thyroid cancers and one of radiation-induced Belarussian and Ukrainian PTC, have concordantly identified the *FOXE1* (*TTF2*) locus as a marker of inherited susceptibility for PTC of different etiology [75,77,78]. The intronless *FOXE1* is a member of the forkhead/winged helix family of evolutionarily conserved transcription factors [79]. In humans, FOXE1 is a key player in thyroid organogenesis, thyrocyte precursors migration and differentiation with onset of expression in the thyroid primordium at Carnegie stage 15 [80,81]. FOXE1 is a transcription activator of thyroperoxidase (*TPO*) and thyroglobulin (*TG*) genes [82,83].

FOXE1 involvement in thyroid diseases remains scarcely addressed. Using RT-PCR or *in situ* hybridization, Sequeira *et al*. found *FOXE1* (*TTF-2*) expression in about 60% of human thyroids [84]. In benign thyroid lesions *FOXE1* expression was observed in 43–100% cases. In thyroid malignancies, *FOXE1* was expressed in 44% follicular carcinomas, 65% PTC and 0 (of 2) anaplastic carcinomas. Nonaka *et al*. reported strong diffuse immunohistochemical TTF-2 staining in 50 – 100% tumor cells in PTC, follicular adenoma, follicular carcinoma and poorly differentiated thyroid carcinoma [85]. Medullary thyroid carcinomas were weakly positive in 75% cases and anaplastic thyroid carcinomas were virtually all negative. In a study by Zhang *et al*., nuclear expression of TTF-2 was found to be gradually decreasing from follicular adenoma to anaplastic carcinoma in accordance with tumor dedifferentiation [86]. Abnormal TTF-2 expression in the cytoplasm displayed the opposite trend except for anaplastic carcinoma in which TTF-2 expression was generally low. Despite genetic studies strongly suggest FOXE1 implication in PTC, and there is an alteration of FOXE1 expression and localization in cancer cells, its role in tumor development remains unknown.

To clarify the role of FOXE1 in PTC, the patterns of FOXE1 immunohistochemical expression in 42 tumors and adjacent normal thyroid were analyzed, and then their relationship with morphological characteristics of the tumor and patients' genotypes was investigated [87].

FOXE1 exhibited nuclear and cytoplasmic staining in normal thyroid follicular cells. Nuclear immunoreactivity was strong or moderate, while cytoplasm showed weak-intensity

staining. Approximately one-third of all nuclei were not stained, whereas no cytoplasmic expression was noticed in about 25% of cells. More uniform pattern of FOXE1 expression was observed in thyroid cells at the vicinity of PTC border within 100-300 μm tissue layer in which most cells showed strong nuclear and moderate cytoplasmic immunoreactivity.

FOXE1 expression in cancer tissue displayed approximately the same extent as in the normal counterpart. Cells at the tumor border and those in close proximity to the border showed the highest intensity of cytoplasmic and nuclear expression. Neoplastic cells in the tumor center exhibited substantially lower intensity scores and an obvious loss of nuclear expression.

A significant increase of total FOXE1 score in cancer tissue compared to normal thyroid ($p<0.001$) which was contributed mainly by the cytoplasmic expression was found while the nuclear expression remained higher in non-neoplastic thyroid cells ($p<0.001$ and $p<0.05$, respectively). Thus, cytoplasmic FOXE1 expression was an essential feature of cancer cells whereas prominent nuclear expression was characteristic for normal cells.

Statistical analysis confirmed preliminary observations of the gradient FOXE1 expression from central to peripheral areas of tumor and normal counterparts. Both cancer and surrounding normal thyroid tissue demonstrated the strongest FOXE1 expression in the areas immediately adjacent to the tumor border comparing with the staining scores of distant regions ($p<0.001$). Of note, the highest nuclear scores were on the normal tissue side and the highest cytoplasmic scores on the cancer side. There were notable changes in FOXE1 immunoreactivity with distance from the tumor border, such as increasing number of negative nuclei in normal thyroid tissue and increasing number of negative nuclei along with decreasing cytoplasmic intensity in cancer tissue (Fig. 7.5).

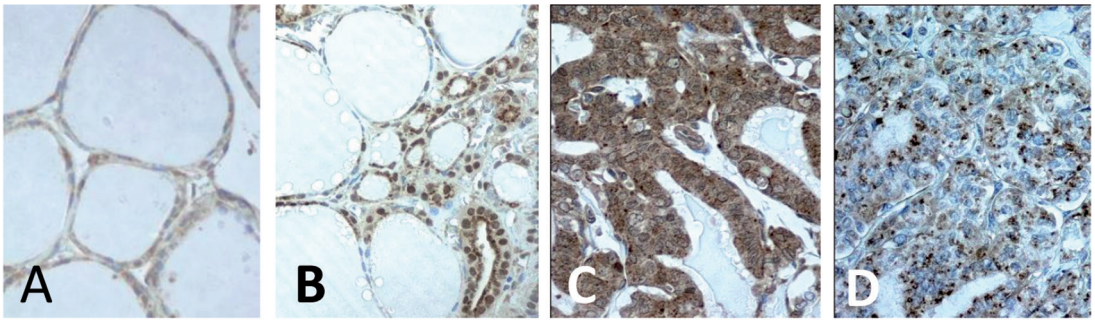

Figure 7.5. Four distinct patterns of immunohistochemical FOXE1 expression in different zones of normal thyroid (A and B) and tumor tissue (C and D), original magnification ×200. (A) In the normal thyroid tissue at the distance >300 μm from the tumor border there is a considerable variation of nuclear and cytoplasmic FOXE1 expression with 25 – 35% FOXE1 negative cells. (B) In the normal thyroid tissue immediately (≤300 μm) adjacent to invasive or encapsulated tumor border) most cells are FOXE1 positive with the highest nuclear and moderate cytoplasmic intensity. (C) In the tumor tissue at the invasive front or subcapsular region (<300 μm) regardless tumor border contacts adjacent thyroid parenchyma or extrathyroid tissues, virtually all cells demonstrate the strongest cytoplasmic and moderate nuclear FOXE1 expression. (D) Tumor tissue at the distance >300 μm from the tumor border shows mainly monomorphic low to moderate cytoplasmic expression with negative or weak nuclear staining.

On multivariate logistic regression analysis it was demonstrated that among all demographic and pathological variables entered in the model, only two, namely tumor multifocality (p=0.032) and rs1867277 polymorphism in the *FOXE1* 5′ UTR (p=0.037), significantly associated with nuclear FOXE1 expression in the zone of tumor cells confined within the 300 μm to the tumor border; capsular invasion displayed marginal significance (p=0.051). Both multifocality and variant genotype other than homozygous for the major allele (the dominant model of inheritance) associated with the higher FOXE1 expression.

Thus, FOXE1 displays an aberrant cytoplasmic expression in PTC suggestive of a possibility of a role other than transcription factor or of the existence of other factors causing translocation; perhaps such translocation may contribute to cancer cell biology. FOXE1 overexpression in the cytoplasm in tumor cells may also reflect activation of FOXE1 regulating pathways (Shh/Gli and Wnt) which are switched on during thyroid carcinogenesis [88]. The precise role of cytoplasmic FOXE1 requires functional studies to determine whether it is an active process involved in carcinogenesis or it is a consequence of malignant transformation.

The interface between normal and cancer tissues is a battlefield of invading cancer cells and the host. Many biological processes in cancer show highest intensity at the border, e.g. cell metabolism changes, proliferation, neovascularization, and invasion. The significant difference of FOXE1 expression between the periphery and the center of PTC is in line with these notions of particular processes at the tumor border. The gradient in non-neoplastic thyroid tissue adjacent to PTC was also found. Such kind of distribution is not described well for normal counterpart. Observations of the sharp reduction of FOXE1-positive cells with the distance from the tumor border imply FOXE1 involvement in communication at the host/tumor interface.

Transcriptomic studies

Gene expression analysis

The previous sections have concentrated on the molecular aberrations identified in the genome. Although many of these will result in changes at the RNA level, other factors can affect RNA expression. The next section therefore summarises data from transcriptomic studies using RNA expression techniques.

The power of the microarray technology in contrast to conventional analysis is in enabling an observation of the genome-wide gene expression profile for a given population within a single experiment. Gene-specific oligonucleotide sequences are spotted on glass slides and used for hybridisation of a preprocessed RNA sample. Technical issues such as non-specific binding of the fluorophore or hybridisation artefacts can influence the evaluation and need to be controlled for. The large amount of data created may be difficult to analyse and needs implementation of explorative statistical tools for normalisation and correction, as well as for detection of significant hits. Gene expression microarray studies are carried out to define diagnostic and prognostic markers and signatures. In Ukrainian post-Chernobyl PTCs, several studies were specifically designed to address the question whether there is a molecular signature for radiation-induced thyroid cancer. Table 7.2 gives an overview of the outcome. No significant findings were reported concerning the transcriptome of exposed and non exposed tumor tissue, but various studies identified subsets of genes involved in thyroid carcinogenesis and their related signalling pathway regulations.

Table 7.2
Overview of conducted gene expression studies in the Ukrainian cohort

Author [ref.]	Hypothesis	Transcriptome	Pathway	Genes
Abend *et al.*, 2012 [103]	dose-response relationship between ^{131}I dose and gene expression	2500 genes differentially expressed in relation to ^{131}I dose	Cell cycle & growth and differentiation, Cell adhesion, Microenvironment/metabolic changes, Transcription factor and DNA modification through methylation ; w/o significant enrichment of a certain pathway	*AJAP1*, *FAM38A*, *CA12*, *LMO3*, *ZNF493*, *MTA1*, *SLC19A1*, *CDK12*, *ACVR2A*, *GALNT7*, *SLC43A3*
Delys *et al.*, 2007 [94]	gene expression is differentially regulated in sporadic/radiation-induced PTC compared to normal thyroid	differetial expression of 44.5% of genes on the array	Immune response, Cytokine activity, Chemokine activity, Thyroid hormone generation, EGFR signaling pathway, Activation of JNK activity , MAP kinase phosphatase activity, Extracellular matrix, Peptidase activity, Protease inhibitor activity, Laminin complex, Collagen, Cell–cell adhesion, Integrin complex; *p*-values below 0.05	*ANXA1*, *CDH3*, *CLDN1*, *DUSP5*, *GPX1*, *HMGA2*, *NELL2*, *NRCAM*, *SLIT1*, *THBS2*, *TNC*, *BCL2*, *EGR1*, *EGR2*, *FLRT2*
Detours *et al.*, 2005 [89]	radiation-signature in post-Chernobyl childhood PTC	no separation of radiation-induced from sporadic PTC	n/a	radiation-induced and sporadic datasets show a high correlation
Detours *et al.*, 2007 [90]	radiation-signature in post-Chernobyl childhood PTC	similar between radiation-induced and sporadic;	n/a	two subsets of 256 and 118 genes separate radiation-induced from sporadic;
			homologous recombination	*XRCC2*, *SHFM1*, *RAD51C*, *MUS81*, *RAD51L1*, *RAD51*, *RAD50*, *RAD54B*, *RAD54L*, *NBS1*, *RAD52*, *XRCC3*, *BRCA1*
Dom *et al.*, 2012 [93]	radiation-signature in post-Chernobyl childhood PTC	separation of normal exposed from normal not exposed tissue	Chronic myeloid leukaemia, Neutrophin signalling pathway, MAPK signalling pathway, Insulin signalling pathway, Renal cell carcinoma, Pancreatic cancer, Regulation of actin cytoskeleton, Spliceosome, mTOR signalling pathway, Apoptosis, Focal adhesion	403 gene signature
Stein *et al.*, 2010 [62]	gene expression changes in radiation-induced childhood PTC in comparison to the matching normal tissue	n/a	Cancer, Cellular Growth and Proliferation, Reproductive System Disease, Cellular Movement, Cell-to-Cell Signaling, and Cell-Mediated Immune Response	*LRP4*, *IGSF1*, *ODZ1*, *CITED1*, *SLIT1*,*HMGA2*, *SERPINA1*, *KCNJ2*, *AGR2*, *TACSTD2*, *NELL2*, *CHI3L1*, *TPO*, *TFF3*, *CRABP1*, *COL9A3*, *MATN2*, *SLC4A4*, *DIO1*, *KIT*, *CCL21*, *FBLN1*, *FHL1*, *MPPED2*

Detours *et al*. [89] performed Micromax Microarray experiments to evaluate the gene expression profile of twelve post-Chernobyl PTCs. In addition, eight sporadic PTCs and thirteen autonomous adenomas from Belgium, France and the US were arrayed. The group was interested whether the gene expression pattern of the radiation exposed cases would reveal a radiation-related signature and applied several unsupervised and supervised analyses to the obtained microarray data. Unsupervised hierarchical clustering was able to distinguish between PTCs and adenomas, but could not separate the sporadic from the post-Chernobyl PTCs. Similarly, *RET/PTC* rearrangement was not a criterion which could separate the PTCs into two groups. Significance analysis of microarray (SAM) found 168 genes to be differentially expressed between adenomas and PTC with a false discovery rate (FDR) of 1%. Amongst these, a signature of six genes was capable of accurately differentiating between adenoma and PTC. No such genes were found when analysing sporadic PTC versus post-chernobyl PTC. Instead, a significant correlation of the two groups was found which could not be seen for the adenomas. This correlation was independent from the methods used and was validated *in silico* using PTC data from Affymetrix microarrays. Patients were not matched on age, residency, or sex within this study. Surprisingly, despite the high variation in the clinical parameters of the patients, the data correlated significantly and indicated a similarity between sporadic and post-Chernobyl cases. The data suggested that no molecular expression signature for radiation-induced thyroid cancerogenesis exists.

Two years later the same group [90] repeated the analysis on Agilent microarrays, which support a broader range of genes. The Ukrainian cohort of twelve patients exposed to radiation was compared to a French cohort of 14 patients without known radiation history. The distribution of histological subtypes was the same in the two groups. Frequencies of *RET/PTC* rearrangement and *BRAF* mutation were also comparable. Screening for global expression profiles revealed no difference between the French and the post-Chernobyl PTCs. However, application of a supervised classification algorithm identified a subset of genes which was capable of distinguishing French and post-Chernobyl tumors with an error rate of 12%. In further experiments *in vitro* the same authors showed that 200 mM H_2O_2 (peroxide) or 2.5 Gy γ-radiation cause a similar transcriptional response in a B lymphocyte cell line with a subset of 293 genes having a fold change greater than 1.5. 118 out of these genes were also able to separate the French and the post-Chernobyl cohort with an error of 15%. This implies that peroxide had a major influence in carcinogenesis in the French cohort. H_2O_2 is a byproduct of thyroid hormone synthesis and known for its high DNA damaging potential due to its oxidizing properties. H_2O_2 is considered as a highly reactive oxygen species, and free radicals were shown to cause cancers in the thyroid of Tg-a1BAR mice [91]. Similarly, ionizing radiation is creating reactive oxygen through the radiolysis of water, which is highly abundant in the cellular environment. The etiological agents peroxide and radiation both create reactive oxygen species as a cancer initiating event which supports the finding of similar global gene expression profiles. An additional signature of thirteen genes involved in homologous recombination was able to distinguish the two cohorts with an error rate of 15%. The authors matched the groups on clinical parameters such as morphology and known molecular aberrations. However, they did not

match on age and residency, two independent confounders known to affect data analysis. It would also have been interesting to see whether the magnitude of *RET/PTC* or *BRAF* expression had an influence on the gene expression profile as described in literature [92], but such data was not provided.

A more recent study again tried to address the question whether a radiation-specific signature is detectable in post-Chernobyl thyroid cancer. Dom *et al*. [93] chose a carefully selected cohort. 22 patients who were exposed to radiation and born before April 1986 were matched on age and residency in Ukraine to 23 patients born after March 1987 and hence not exposed to radiation. The cases were subjected to Affymetrix microarrays and expression of single genes was validated using qRT-PCR.

The authors searched for global gene expression variations using hierarchical clustering and principal components analysis which successfully separated tumor from normal thyroid. However, similar analyses were not able to reveal any differences between exposed and non exposed cases. A subset of eight genes was selected according to known involvement of their products in carcinogenesis: carbonic anhydrase 12, BH3-interacting domain death agonist, clusterin, cyclin D2, trefoil factor 3, low-density lipoprotein receptor-related protein 1B, dual specificity phosphatase 1 (DUSP1) and thrombospondin, type I, domain-containing 7A, and validated by qRT-PCR. All of them were found to be differentially regulated in tumor versus normal thyroid tissue.

Supervised analysis of the data could not distinguish exposed from non exposed tumors either, but the normal tissue seemed to have differential gene expression in both groups.

Potential confounding factors such as the clinical parameters sex, age at operation, patients' place of residence (region/oblast) at the time point of the Chernobyl accident, morphological subtype of PTC, TNM classification, presence of *BRAF* mutations or *RET/PTC* rearrangements, tumor size and methodological parameters such as batch effects of microarray hybridisation or RNA storage time were analysed. The age at operation and the time of storage of frozen tissue prior to RNA extraction were found to be associated with the radiation exposure, and data was adjusted accordingly. The corrected data was evaluated using SAM and a signature of 403 genes was found to distinguish exposed from non exposed normal thyroid tissue. All of them were upregulated in the exposed normal tissue. The results were validated *in silico* using an external data set. Gene ontology and KEGG analysis of the gene signature in DAVID found pathways with an impact on cancer development and proliferation, mainly MAPK, insulin and mTOR signalling pathways, and enrichment of cell adhesion-related genes.

Thus, the study was not able to show any differences between exposed and non exposed tumors, but the matching contralateral normal tissue in these patients reveals significant differences. This could be related to the character of the tumor which is known to evolve into diverse subpopulations, whereas the surrounding normal tissue does not evolve but may keep a tumor-initiating expression profile. However, such hypotheses remain speculative and require validation in an independent set of cases. The signature identified could be used to classify a PTC cohort in the two categories - exposed and non exposed, but was associated with a false positive rate of 30%.

Pathway regulation

Although scientists could not detect a clear radiation-related signature in post-Chernobyl childhood PTC, with the methods used, the collected data highlighted certain signalling pathways in exposed over unexposed tumor tissue. The point mutations and gene rearrangements observed in PTC stress out a major role for the MAPK pathway in thyroid tumorigenesis. *BRAF* mutations and *RET* as well as *NTRK* rearrangements lead to constitutive activation of these genes and are supposed to interfere with their downstream partners perturbing the pathway cascade. Expression status of other genes - especially in post-Chernobyl PTC - was rarely explored. The study of Delys *et al*. [94] aimed to identify certain signalling pathways and their regulation in relation to the physiopathology of the disease. 12 PTCs from Ukrainian patients which were exposed to radiation and 15 sporadic PTCs from French patients plus matching normal tissues were arrayed on Agilent cDNA microarrays. The statistical algorithm Significance Analysis of Microarray (SAM) was applied, and it was possible to significantly distinguish a characteristic gene expression profile for PTC from normal thyroid tissue. The differences in gene expression may relate to the observed differences in morphology between tumor and normal tissue. To identify individual differentially regulated genes, the data was correlated with two independent PTC data sets [95,96]. Only genes which show the same regulation in at least two of the data sets were selected for further pathway regulation studies. This resulted in a profile of 451 up- and 233 downregulated genes. Eleven upregulated genes (*ANXA1, CDH3, CLDN1, DUSP5, GPX1, HMGA2, NELL2, NRCAM, SLIT1, THBS2, TNC*) and four downregulated genes (*BCL2, EGR1, EGR2, FLRT2*) identified in the microarray data sets were validated by qRT-PCR. Additionally, 20 genes were already identified in the literature to correlate with the phenotype of sporadic thyroid cancer [97]. GO term analysis with DAVID revealed a participation of the genes in the gene list in eleven pathways with a p value <0.05. Many of the pathways are related to the immune response which may more reflect the infiltration of immune cells into the tumor rather than changes in the tumor cell itself. Alteration of cytokines and chemokines was observed in this study as well, which is consistent with other studies where PTC is known to evoke a chronic inflammation *in vivo* and *in vitro* [98]. This could have a direct influence on the recruitment of lymphatic cells into the tumor tissue.

Surprisingly, no deregulation of the MAPK pathway could be found, which would be expected for PTC because of the *BRAF* mutations and *RET/PTC* gene rearrangements which lead to a constitutive expression of those genes. On the other hand, EGF signalling, an activator of the MAPK pathway, was significantly altered, whereas ERK specific inhibitors of the DUSP family were highly overexpressed as well. c-jun N-terminal kinase (JNK) and p38 pathway was another gene cascade significantly altered. Its involvement in thyroid carcinogenesis was already suggested previously by immunohistochemical detection in tumor but not normal thyroid tissue [99]. Also, overexpression of proteases and protease inhibitors which are known for remodelling of the ECM was highly abundant in PTC versus normal tissue. This is a well known cancer initiating event and documented to have a participation in thyroid cancer [100,101].

Cell migration is another mechanism playing an important role in carcinogenesis. Its consequences for tumor development can be observed and are described histopathologically [102]. Delys *et al*. [94] identified several genes from the integrin, cadherin and claudin family being deregulated in the tumor tissue, which could have a direct influence on the invasive behaviour of the tumor. It would be interesting to apply a principal component analysis to these 26 cases of the study to see whether clinical parameters match with these findings, and tumors with a higher invasive behaviour consistently show a stronger upregulation of these pathways.

The study could identify a clear difference between PTC and normal tissue and found many pathways which are known to be involved in tumor development being differentially regulated. The study could not distinguish between radiation-induced and sporadic tumors, suggesting that the etiologic agent may be responsible for tumor initiation but has no influence on tumor progression.

Transcriptomic profiles and radiation dose

Previous studies showed that, especially in gene expression analysis, the use of differing methods and statistical evaluation algorithms, the sample size and/or selection of control populations lead to inconsistent findings between studies. These factors may have contributed to the conclusion that neither a radiation-specific signature nor a gene regulation mechanism is present in the Ukrainian post-Chernobyl PTC cases. Despite this, a more recent study performed by Abend *et al*. [103] aimed to address the question whether a dose-related response to ^{131}I is present and detectable. The patients included in this study were enrolled in the Ukraine-American Cohort Study. The patients were all below 18 years old at the time of accident and individual ^{131}I measurements were taken within two months after the accident [104]. 63 tumor/normal pairs were split in a screening set with 32 cases for Agilent oligo microarray gene expression analysis and a validation set with 31 cases for qRT-PCR validation of altered genes. Arraying found 2500 genes that were significantly differentially expressed between the tumor and the normal tissue, but none of the *p* values survived multiple testing using false discovery rate (FDR). 75 genes were selected based on analysis of a dose-dependent differential expression, and this list includes also genes with evidence from previous studies. qRT-PCR found 11 of those genes were differentially expressed in tumor but not in normal tissue in relation to the dose received. Nine of the eleven genes are involved in the pathways cell adhesion (*AJAP1, FAM38A*), energy metabolism (*CA12*), transcription or DNA methylation (*LMO3, ZNF493, MTA1, SLC19A1*), and growth/differentiation (*CDK12, ACVR2A*). These pathways are known for their participation in the cellular response to ionizing radiation [105, 106]. The importance of five genes (*CA12, GALNT7, LMO3, SLC43A3 and FAM38A*) was also confirmed by others in post-Chernobyl and other radiation-induced childhood thyroid tumors [62, 107]. The differential expression of the genes is dose-dependent and linear, however, the three genes *SLC19A1, SLC43A3* and *ZNF493* exhibit some non-linearity. The cellular response to radiation is known to be non-linear, and changes over time with an early response to DNA damage and a later survival with chromosomal rearrangements [108,109], and the non-linearity in differential expression may reflect this. Taken together, the eleven genes show

a clear difference in expression levels between exposed tumor and exposed normal tissue, and further studies on the genomic architecture of these genes will give more insight on their ability to serve as biomarkers for radiation exposure.

In the most recently reported study using material from the patients enrolled in the Ukrainian-American cohort, Leeman-Neill *et al*. [110] hypothesized that chromosomal aberrations such as *RET/PTC* and point mutations have different associations with ^{131}I dose. The case series included 26 males (42%) and 36 females (58%) who were resident in the areas surrounding Chernobyl, i.e., the Zhytomir (n= 17; 27.4%), Kiev (n=12; 19.4%), or Chernigov (n=33; 53.2%) regions (oblasts), and who were between 5 months and 17 years old (mean 8.0 years) at the time of the Chernobyl accident. The estimated ^{131}I dose for patients in the study ranged from 0.008 Gy to 8.6 Gy, with a mean dose of 1.3 Gy. Surgical removal of PTCs occurred between October 1998 and December 2007, with time between exposure and surgery ranging from 12.5 to 21.6 years (mean: 16.5 years).

RET/PTC rearrangement was the most common genetic alteration and was found in 22 (35%) cases, including 14 *RET/PTC1* and 8 *RET/PTC3* rearrangements. Point mutations in the *BRAF* and *RAS* genes were found in 9 (15%) and 5 (8%) of the tumors, respectively. All 9 *BRAF* mutations were *BRAF*^{V600E}. Four *RAS* mutations were detected in *NRAS* codon 61 and 1 in *HRAS* codon 61. No *KRAS* mutations were found in codons 12 and 13. In addition, these tumors were studied for *PAX8/PPAR*γ rearrangement, a prototypic genetic alteration found in follicular thyroid carcinoma that occurs with lower prevalence in the follicular variant of PTC. Two tumors were positive for *PAX8/PPAR*γ; both were of the follicular variant of PTC. In both cases, the fusions were between exon 9 of *PAX8* and exon 1 of *PPAR*γ, with several expected splice variants of the chimeric *PAX8/PPAR*γ transcripts detected. One tumor had more than 1 mutation, harbouring an *NRAS* point mutation in codon 61 and *PAX8/PPAR*γ rearrangement. Twenty five (40%) tumors revealed none of the studied mutations. Patients with *BRAF* or *RAS* mutations had the lowest dose (0.27 Gy and 0.20 Gy, respectively). *RET/PTC1* and *RET/PTC3* were associated with a higher dose of 1.04 Gy and 1.54 Gy. The highest dose of 1.97 Gy had tumors without any of the observed aberrations. There was also a significant correlation with the age of the patients, where *BRAF* and *RAS* mutations associated with older age and *RET/PTC* rearrangements with younger onset. Hence, it is difficult to differentiate between the known effects of age at diagnosis on the molecular phenotype of PTC, and the effects of dose, especially in such a small study. The cohort was not age matched or subdivided between children and adolescents (aged <19) and young adults (aged >19) at surgery. Children have been shown to receive a relatively higher dose of radiation due to the smaller size of their thyroid.

Interestingly, there was a positive correlation of the chromosomal rearrangements with residency in the Zhytomir oblast, where the individuals also received a higher radiation dose. This oblast is known for a moderate iodine deficiency. As such a deficiency was shown to be a risk factor for thyroid cancer in a Belarussian cohort of post-Chernobyl thyroids [111], the authors suggest a similar contribution for their findings.

Further whole genome studies on cohorts matched for the confounders in this study (age, sex, region of residence) would be required to convincingly link the findings in this paper and that by Abend *et al*. [103] to dose of radiation.

In summary, it is now accepted that the initial studies suggesting that post- Chernobyl thyroid cancers showed a higher frequency of involvement of the *RET/PTC* oncogene were misinterpreted due to the lack of appropriate age-matched controls. It had been assumed that the molecular pathology of thyroid cancer was not influenced by age, but as the studies reported above have shown, young onset PTC shows very different pathology and morphology when compared with the same disease in adults. Studies using the newer "omic" technologies have suggested that there may be subtle differences between PTCs of a radiation aetiology compared with age-matched controls, but all of these studies require validation in separate cohorts of patients. The studies so far performed on the effect of inherited, germline polymorphisms suggest, as with their somatic counterparts, that there is little to differentiate radiation associated PTC from sporadic PTC. Attention is now turning to studies on potential epigenetic differences (miRNA and DNA methylation), and to next generation sequencing technologies to tease out more subtle differences associated with a radiation aetiology. Regardless of the final conclusions of these studies regarding the relationship between radiation and molecular phenotype, the Ukrainian cases provided to the general scientific community through the generosity of Ukrainian patients and the staff at the Institute of Endocrinology and Metabolism in Kiev, have been instrumental in increasing our knowledge of molecular pathology of thyroid cancer in general, and PTC in particular. It remains to be seen whether any of the data so far generated by these studies, and those yet to come, will lead to the development of better prognostic markers for thyroid cancer. Unlike many cancers, PTC has a releatively good prognosis, with a predicted death rate over 30-50 years of 1%. Despite this, there is a recurrence rate of 30% [112]. Identification of prognostic biomarkers, combined with clinical features would therefore aid patient stratification for follow-up.

References

1. Cancer incidence in five continents. Volume V. *IARC scientific publications;* 1987. pp. 1-970.
2. Williams ED, Abrosimov A, Bogdanova T, *et al.* Morphologic characteristics of Chernobyl-related childhood papillary thyroid carcinomas are independent of radiation exposure but vary with iodine intake. *Thyroid* 2008;18:847-852.
3. Robinson MJ and Cobb MH. Mitogen-activated protein kinase pathways. *Current Opin Cell Biol* 1997;9:180-186.
4. Ciampi R, Knauf JA, Rabes HM, *et al.* BRAF kinase activation via chromosomal rearrangement in radiation-induced and sporadic thyroid cancer. *Cell Cycle* 2005;4:547-548.
5. Soares P, Trovisco V, Rocha AS, *et al.* BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. *Oncogene* 2003;22:4578-4580.
6. Frattini M, Ferrario C, Bressan P, *et al.* Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* 2004;23:7436-7440.

7. Roque L, Nunes VM, Ribeiro C, *et al.* Karyotypic characterization of papillary thyroid carcinomas. *Cancer* 2001;92:2529-2538.
8. Bongarzone I, Fugazzola L, Vigneri P, *et al.* Age-related activation of the tyrosine kinase receptor protooncogenes RET and NTRK1 in papillary thyroid carcinoma. *J Clin Endocrinol Metab* 1996;81:2006-2009.
9. Said S, Schlumberger M and Suarez HG. Oncogenes and anti-oncogenes in human epithelial thyroid tumors. *J Endocrinol Invest* 1994;17:371-379.
10. Olah E, Balogh E, Bojan F, *et al.* Cytogenetic analyses of three papillary carcinomas and a follicular adenoma of the thyroid. *Cancer Genet Cytogenet* 1990;44:119-129.
11. Nikiforova MN, Stringer JR, Blough R, *et al.* Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. *Science* 2000;290:138-141.
12. Yang T, Namba H, Hara T, *et al.* p53 induced by ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells. *Oncogene* 1997;14:1511-1519.
13. Jhiang SM. The RET proto-oncogene in human cancers. *Oncogene* 2000;19:5590-5597.
14. Knauf JA, Kuroda H, Basu S, *et al.* RET/PTC-induced dedifferentiation of thyroid cells is mediated through Y1062 signaling through SHC-RAS-MAP kinase. *Oncogene* 2003;22:4406-4412.
15. Santoro M, Carlomagno F, Hay ID, *et al.* Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. *J Clin Invest* 1992;89:1517-1522.
16. Tallini G, Santoro M, Helie M, *et al.* RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. *Clin Cancer Res* 1998;4:287-294.
17. Zhu Z, Ciampi R, Nikiforova MN, *et al.* Prevalence of RET/PTC rearrangements in thyroid papillary carcinomas: effects of the detection methods and genetic heterogeneity. *J Clin Endocrinol Metab* 2006;91:3603-3610.
18. Ito T, Seyama T, Iwamoto KS, *et al.* Activated RET oncogene in thyroid cancers of children from areas contaminated by Chernobyl accident. *Lancet* 1994;344:259.
19. Fugazzola L, Pilotti S, Pinchera A, *et al.* Oncogenic rearrangements of the RET proto-oncogene in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear accident. *Cancer Res* 1995;55:5617-5620.
20. Klugbauer S, Lengfelder E, Demidchik EP, *et al.* High prevalence of RET rearrangement in thyroid tumors of children from Belarus after the Chernobyl reactor accident. *Oncogene* 1995;11: 2459-2467.
21. Santoro M, Thomas GA, Vecchio G, *et al.* Gene rearrangement and Chernobyl related thyroid cancers. *Br J Cancer* 2000;82:315-322.
22. Nikiforov YE, Rowland JM, Bove KE, *et al.* Distinct pattern of ret oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. *Cancer Res* 1997;57:1690-1694.
23. Jhiang SM, Sagartz JE, Tong Q, *et al.* Targeted expression of the ret/PTC1 oncogene induces papillary thyroid carcinomas. *Endocrinology* 1996;137:375-378.
24. Powell DJ, Jr., Russell J, Nibu K, *et al.* The RET/PTC3 oncogene: metastatic solid-type papillary carcinomas in murine thyroids. *Cancer Res* 1998;58:5523-5528.

25. Thomas GA, Bunnell H, Cook HA, *et al.* High prevalence of RET/PTC rearrangements in Ukrainian and Belarussian post-Chernobyl thyroid papillary carcinomas: a strong correlation between RET/PTC3 and the solid-follicular variant. *J Clin Endocrinol Metab* 1999;84:4232-4238.
26. Bounacer A, Wicker R, Caillou B, *et al.* High prevalence of activating ret proto-oncogene rearrangements, in thyroid tumors from patients who had received external radiation. *Oncogene* 1997;15:1263-1273.
27. Ito T, Seyama T, Iwamoto KS, *et al.* In vitro irradiation is able to cause RET oncogene rearrangement. *Cancer Res* 1993;53:2940-2943.
28. Furmanchuk AW, Averkin JI, Egloff B, *et al.* Pathomorphological findings in thyroid cancers of children from the Republic of Belarus: a study of 86 cases occurring between 1986 ('post-Chernobyl') and 1991. *Histopathology* 1992;21:401-408.
29. Fenton CL, Lukes Y, Nicholson D, *et al*, The ret/PTC mutations are common in sporadic papillary thyroid carcinoma of children and young adults. *The J Clin Endocrinol Metab* 2000;85:1170-1175.
30. Powell N, Jeremiah S, Morishita M, *et al.* Frequency of BRAF T1796A mutation in papillary thyroid carcinoma relates to age of patient at diagnosis and not to radiation exposure. *J Pathol* 2005;205:558-564.
31. Unger K, Zitzelsberger H, Salvatore G, *et al.* Heterogeneity in the distribution of RET/PTC rearrangements within individual post-Chernobyl papillary thyroid carcinomas. *J Clin Endocrinol Metab* 2004;89:4272-4279.
32. Unger K, Zurnadzhy L, Walch A, *et al.* RET rearrangements in post-Chernobyl papillary thyroid carcinomas with a short latency analysed by interphase FISH. *Br J Cancer* 2006;94:1472-1477.
33. Williams ED, Abrosimov A, Bogdanova T, *et al.* Thyroid carcinoma after Chernobyl latent period, morphology and aggressiveness. *Br J Cancer* 2004;90:2219-2224.
34. Hieber L, Huber R, Bauer V, *et al.* Chromosomal rearrangements in post-Chernobyl papillary thyroid carcinomas: evaluation by spectral karyotyping and automated interphase FISH. *J Biomed Biotechnol* 2011;2011:693691.
35. Tuttle RM, Lukes Y, Onstad L, *et al.* ret/PTC activation is not associated with individual radiation dose estimates in a pilot study of neoplastic thyroid nodules arising in Russian children and adults exposed to Chernobyl fallout. *Thyroid* 2008;18:839-846.
36. Cohen Y, Xing M, Mambo E, *et al.*, BRAF mutation in papillary thyroid carcinoma. *J Natl Cancer Inst* 2003;95:625-627.
37. Kimura ET, Nikiforova MN, Zhu Z, *et al.* High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Res* 2003;63:1454-1457.
38. Xing M, Alzahrani AS, Carson KA, *et al.* Association between BRAF V600E mutation and mortality in patients with papillary thyroid cancer. *JAMA* 2013;309:1493-1501.
39. Kumagai A, Namba H, Saenko VA, *et al.* Low frequency of BRAFT1796A mutations in childhood thyroid carcinomas. *J Clin Endocrinol Metab* 2004;89:4280-4284.
40. Lima J, Trovisco V, Soares P, *et al*, BRAF mutations are not a major event in post-Chernobyl childhood thyroid carcinomas. *J Clin Endocrinol Metab* 2004;89:4267-4271.

41. Bongarzone I, Pierotti MA, Monzini N, *et al.* High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. *Oncogene* 1989;4:1457-1462.
42. Pierotti MA. Chromosomal rearrangements in thyroid carcinomas: a recombination or death dilemma. *Cancer Let* 2001;166:1-7.
43. Beimfohr C, Klugbauer S, Demidchik EP, *et al.* NTRK1 re-arrangement in papillary thyroid carcinomas of children after the Chernobyl reactor accident. *Int J Cancer* 1999;80:842-847.
44. Rabes HM, Demidchik EP, Sidorow JD, *et al.* Pattern of radiation-induced RET and NTRK1 rearrangements in 191 post-chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. *Clin Cancer Res* 200;6:1093-1103.
45. Alberti L, Carniti C, Miranda C, *et al.* RET and NTRK1 proto-oncogenes in human diseases. *J Cell Physiol* 2003;195:168-186.
46. Pierotti MA and Greco A. Oncogenic rearrangements of the NTRK1/NGF receptor. *Cancer Let* 2006;232:90-98.
47. Lemoine NR, Mayall ES, Wyllie FS, *et al.* High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene* 1989;4:159-164.
48. Ito T, Seyama T, Mizuno T, *et al.* Unique association of p53 mutations with undifferentiated but not with differentiated carcinomas of the thyroid gland. *Cancer Res* 1992;52:1369-1371.
49. Parma J, Van Sande J, Swillens S, *et al.* Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: identification of additional mutations activating both the cyclic adenosine 3',5'-monophosphate and inositol phosphate-Ca2+ cascades. *Mol Endocrinol* 1995;9:725-733.
50. Russo D, Arturi F, Schlumberger M, *et al.* Activating mutations of the TSH receptor in differentiated thyroid carcinomas. *Oncogene* 1995;11:1907-1911.
51. Hillebrandt S, Streffer C, Demidchik EP, *et al.* Polymorphisms in the p53 gene in thyroid tumors and blood samples of children from areas in Belarus. *Mutation Res* 1997;381:201-207.
52. Smida J, Zitzelsberger H, Kellerer AM, *et al.* p53 mutations in childhood thyroid tumors from Belarus and in thyroid tumors without radiation history. *Int J Cancer* 1997;73:802-807.
53. Suchy B, Waldmann V, Klugbauer S, *et al.* Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumors. *Br J Cancer* 1998;77:952-955.
54. Nikiforov YE, Nikiforova MN, Gnepp DR, *et al.* Prevalence of mutations of ras and p53 in benign and malignant thyroid tumors from children exposed to radiation after the Chernobyl nuclear accident. *Oncogene* 1996;13:687-693.
55. Fenton C, Anderson J, Lukes Y, *et al.* Ras mutations are uncommon in sporadic thyroid cancer in children and young adults. *J Endocrinol Invest* 1999;22:781-789.
56. Unger K, Malisch E, Thomas G, *et al.* Array CGH demonstrates characteristic aberration signatures in human papillary thyroid carcinomas governed by RET/PTC. *Oncogene* 2008;27:4592-4602.
57. Kadota M, Tamaki Y, Sekimoto M, *et al.* Loss of heterozygosity on chromosome 16p and 18q in anaplastic thyroid carcinoma. *Oncol Rep* 2003;10:35-38.
58. Kitamura Y, Shimizu K, Nagahama M, *et al.* Immediate causes of death in thyroid carcinoma: clinicopathological analysis of 161 fatal cases. *J Clin Endocrinol Metab* 1999;84:4043-4049.

59. Singh B, Lim D, Cigudosa JC, *et al.* Screening for genetic aberrations in papillary thyroid cancer by using comparative genomic hybridization. *Surgery* 2000;128:888-893;discussion:893-884.

60. Finn SP, Smyth P, O'Regan E, *et al.* Array comparative genomic hybridisation analysis of gamma-irradiated human thyrocytes. *Virch Archiv* 2004;445:396-404.

61. Richter H, Braselmann H, Hieber L, *et al.* Chromosomal imbalances in post-chernobyl thyroid tumors. *Thyroid* 2004;14:1061-1064.

62. Stein L, Rothschild J, Luce J, *et al.* Copy number and gene expression alterations in radiation-induced papillary thyroid carcinoma from chernobyl pediatric patients. *Thyroid* 2010,20:475-487.

63. Bauer AJ, Cavalli LR, Rone JD, *et al.* Evaluation of adult papillary thyroid carcinomas by comparative genomic hybridization and microsatellite instability analysis. *Cancer Genet Cytogenet* 2002;135:182-186.

64. Rodrigues R, Roque L, Espadinha C, *et al.* Comparative genomic hybridization, BRAF, RAS, RET, and oligo-array analysis in aneuploid papillary thyroid carcinomas. *Oncol Rep* 2007;18:917-926.

65. Kjellman P, Lagercrantz S, Hoog A, *et al.* Gain of 1q and loss of 9q21.3-q32 are associated with a less favorable prognosis in papillary thyroid carcinoma. *Genes Chromosomes Cancer* 2001; 32:43-49.

66. Hess J, Thomas G, Braselmann H, *et al.* Gain of chromosome band 7q11 in papillary thyroid carcinomas of young patients is associated with exposure to low-dose irradiation. *Proceedings Natl Acad Sciences USA* 2011;108:9595-9600.

67. Hemmer S, Wasenius VM, Knuutila S, *et al.* DNA copy number changes in thyroid carcinoma. *Am J Pathol* 1999;154:1539-1547.

68. Perissel B, Coupier I, De Latour M, *et al.* Structural and numerical aberrations of chromosome 22 in a case of follicular variant of papillary thyroid carcinoma revealed by conventional and molecular cytogenetics. *Cancer Genet Cytogenet* 2000;121:33-37.

69. Kitamura Y, Shimizu K, Tanaka S, *et al.* Association of allelic loss on 1q, 4p, 7q, 9p, 9q, and 16q with postoperative death in papillary thyroid carcinoma. *Clin Cancer Res* 2000;6:1819-1825.

70. Stephens LA, Powell NG, Grubb J, *et al.* Investigation of loss of heterozygosity and SNP frequencies in the RET gene in papillary thyroid carcinoma. *Thyroid* 2005;15:100-104.

71. Kimmel RR, Zhao LP, Nguyen D, *et al.* Microarray comparative genomic hybridization reveals genome-wide patterns of DNA gains and losses in post-hernobyl thyroid cancer. *Rad Res* 2006;166:519-531.

72. Rogounovitch TI, Saenko VA, Ashizawa K, *et al.* TP53 codon 72 polymorphism in radiation-associated human papillary thyroid cancer. *Oncol Rep* 2006;15:949-956.

73. Ivanov VK, Gorski AI, Maksioutov MA, *et al.* Thyroid cancer incidence among adolescents and adults in the Bryansk region of Russia following the Chernobyl accident. *Health Physics* 2003;84:46-60.

74. Akulevich NM, Saenko VA, Rogounovitch TI, *et al.* Polymorphisms of DNA damage response genes in radiation-related and sporadic papillary thyroid carcinoma. *Endocr Related Cancer* 2009;16:491-503.

75. Gudmundsson J, Sulem P, Gudbjartsson DF, *et al.* Common variants on 9q22.33 and 14q13.3 predispose to thyroid cancer in European populations. *Nat Genet* 2009;41:460-464.

76. Gudmundsson J, Sulem P, Gudbjartsson DF, *et al.* Discovery of common variants associated with low TSH levels and thyroid cancer risk. *Nat Genet* 2012;44:319-322.

77. Landa I, Ruiz-Llorente S, Montero-Conde C, *et al.* The variant rs1867277 in FOXE1 gene confers thyroid cancer susceptibility through the recruitment of USF1/USF2 transcription factors. *PLoS Genet* 2009;5:e1000637.

78. Takahashi M, Saenko VA, Rogounovitch TI, *et al.* The FOXE1 locus is a major genetic determinant for radiation-related thyroid carcinoma in Chernobyl. *Hum Mol Genet* 2010;19:2516-2523.

79. Chadwick BP, Obermayr F and Frischauf AM FKHL15, a new human member of the forkhead gene family located on chromosome 9q22. *Genomics* 1997;41:390-396.

80. Trueba SS, Auge J, Mattei G, *et al.* PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. *J Clin Endocrinol Metab* 2005;90:455-462.

81. Zannini M, Avantaggiato V, Biffali E, *et al.* TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation. *EMBO J* 1997;16:3185-3197.

82. Aza-Blanc P, Di Lauro R and Santisteban P. Identification of a cis-regulatory element and a thyroid-specific nuclear factor mediating the hormonal regulation of rat thyroid peroxidase promoter activity. *Mol Endocrinol* 1993;7:1297-1306.

83. Civitareale D, Saiardi A and Falasca P. Purification and characterization of thyroid transcription factor 2. *Biochem J* 1994;304:981-985.

84. Sequeira MJ, Morgan JM, Fuhrer D, *et al.* Thyroid transcription factor-2 gene expression in benign and malignant thyroid lesions. *Thyroid* 2001;11:995-1001.

85. Nonaka D, Tang Y, Chiriboga L, *et al.* Diagnostic utility of thyroid transcription factors Pax8 and TTF-2 (FoxE1) in thyroid epithelial neoplasms. *Mod Pathol* 2008;21:192-200.

86. Zhang P, Zuo H, Nakamura Y, *et al.* Immunohistochemical analysis of thyroid-specific transcription factors in thyroid tumors. *Pathol Int* 2006;56:240-245.

87. Bychkov A, Saenko V, Nakashima M, *et al.* Patterns of FOXE1 expression in papillary thyroid carcinoma by immunohistochemistry. *Thyroid* 2013; 23:817-828.

88. Choi JH, Kim BK, Kim JK, *et al.* Downregulation of Foxe1 by HR suppresses Msx1 expression in the hair follicles of Hr(Hp) mice. *BMB Rep* 2011;44:478-483.

89. Detours V, Wattel S, Venet D, *et al.* Absence of a specific radiation signature in post-Chernobyl thyroid cancers. *Br J Cancer* 2005;92:1545-1552.

90. Detours V, Delys L, Libert F, *et al.* Genome-wide gene expression profiling suggests distinct radiation susceptibilities in sporadic and post-Chernobyl papillary thyroid cancers. *Br J Cancer* 2007;97:818-825.

91. Ledent C, Denef JF, Cottecchia S, *et al.* Costimulation of adenylyl cyclase and phospholipase C by a mutant alpha 1B-adrenergic receptor transgene promotes malignant transformation of thyroid follicular cells. *Endocrinology* 1997;138:369-378.

92. Giordano TJ, Kuick R, Thomas DG, *et al.* Molecular classification of papillary thyroid carcinoma: distinct BRAF, RAS, and RET/PTC mutation-specific gene expression profiles discovered by DNA microarray analysis. *Oncogene* 2005;24:6646-6656.

93. Dom G, Tarabichi M, Unger K, *et al.* A gene expression signature distinguishes normal tissues of sporadic and radiation-induced papillary thyroid carcinomas. *Br J Cancer* 2012;107:994-1000.

94. Delys L, Detours V, Franc B, *et al.* Gene expression and the biological phenotype of papillary thyroid carcinomas. *Oncogene* 2007;26:7894-7903.

95. Jarzab B, Wiench M, Fujarewicz K, *et al.* Gene expression profile of papillary thyroid cancer: sources of variability and diagnostic implications. *Cancer Res* 2005;65:1587-1597.

96. Huang Y, Prasad M, Lemon WJ, *et al.* Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proceedings Natl Acad Sciences USA* 2001;98:15044-15049.

97. Kondo T, Ezzat S and Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat Rev Cancer* 2006;6:292-306.

98. Guarino V, Castellone MD, Avilla E, *et al.* Thyroid cancer and inflammation. *Mol Cell Endocrinol* 2010;321:94-102.

99. Shin E, Hong SW, Kim SH, *et al.* Expression of down stream molecules of RET (p-ERK, p-p38 MAPK, p-JNK and p-AKT) in papillary thyroid carcinomas. *Yonsei Med J* 2004;45:306-313.

100. Skrzydlewska E, Sulkowska M, Koda M, *et al.* Proteolytic-antiproteolytic balance and its regulation in carcinogenesis. *World J Gastroenterol* 2005;11:1251-1266.

101. Sid B, Dedieu S, Delorme N, *et al.* Human thyroid carcinoma cell invasion is controlled by the low density lipoprotein receptor-related protein-mediated clearance of urokinase plasminogen activator. *Int J Biochem Cell Biol* 2006;38:1729-1740.

102. Friedl P. Prespecification and plasticity: shifting mechanisms of cell migration. *Current opin Cell Biol* 2004;16:14-23.

103. Abend M, Pfeiffer RM, Ruf C, *et al.* Iodine-131 dose dependent gene expression in thyroid cancers and corresponding normal tissues following the Chernobyl accident. *PloS ONE* 2012;7:e39103.

104. Stezhko VA, Buglova EE, Danilova LI, *et al.* A cohort study of thyroid cancer and other thyroid diseases after the Chornobyl accident: objectives, design and methods. *Rad Res* 2004;161: 481-492.

105. Goldberg Z, Rocke DM, Schwietert C, *et al.* Human in vivo dose-response to controlled, low-dose low linear energy transfer ionizing radiation exposure. *Clin Cancer Res* 2006;12:3723-3729.

106. Wyrobek AJ, Manohar CF, Krishnan VV, *et al.* Low dose radiation response curves, networks and pathways in human lymphoblastoid cells exposed from 1 to 10cGy of acute gamma radiation. *Mutation Res* 2011;722:119-130.

107. Ory C, Ugolin N, Levalois C, *et al.* Gene expression signature discriminates sporadic from post-radiotherapy-induced thyroid tumors. *Endocr Related Cancer* 2011;18:193-206.

108. Romm H and Stephan G. Dose dependency of FISH-detected translocations in stable and unstable cells after Cs gamma irradiation of human lymphocytes in vitro. *Cytogenetic Genome Res* 2004;104:162-167.

109. Caudill CM, Zhu Z, Ciampi R, *et al.* Dose-dependent generation of RET/PTC in human thyroid cells after in vitro exposure to gamma-radiation: a model of carcinogenic chromosomal rearrangement induced by ionizing radiation. *J Clin Endocrinol Metab* 2005;90:2364-2369.

110. Leeman-Neill RJ, Brenner AV, Little MP, *et al.* RET/PTC and PAX8/PPARgamma chromosomal rearrangements in post-Chernobyl thyroid cancer and their association with iodine-131 radiation dose and other characteristics. *Cancer* 2013;119:1792-1799.

111. Cardis E, Howe G, Ron E, *et al.* Cancer consequences of the Chernobyl accident: 20 years on. *J Rad Prot* 2006;26:127-140.

112. Tuttle RM, Vaisman F and Tronko MD. Clinical presentation and clinical outcomes in Chernobyl-related paediatric thyroid cancers: what do we know now? What can we expect in the future? *Clin Oncol* 2011;23:268-275.