Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
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Chapter 6

Molecular-Cytogenetic Characterization of Chernobyl-associated Thyroid Neoplasia in Children


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Abstract

**Background:** Although the 1986 Chernobyl nuclear power plant accident helped to solidify the causal relationship between radiation exposure and development of thyroid neoplasia, the molecular pathogenesis underlying the increase in tumorigenesis remains ill defined. We assessed the contribution of chromosomal instability and abrogation in the components of the p53 pathway to the pathogenesis of Chernobyl-associated thyroid neoplasms in patients from the Ukraine and The Russian Federation presenting during the period between 1995 and 1998.

**Methods:** Chromosomal aberrations were investigated in 28 Chernobyl-associated papillary thyroid carcinoma samples using comparative genomic hybridization (CGH). The expression of p53, MDM2, p21, Bcl-2, Cyclin D1 and Ki-67 was assessed in 73 Chernobyl-associated thyroid tumors arrayed on tissue microarrays (TMA) by immunohistochemistry.

**Results:** Chromosomal instability was detected in 2 of 28 PTC (7%). Chromosomal aberrations included losses of chromosomal regions 6q12-15, 13q21-31, 16q22-24 and Xq1-26. p53 expression was not detected in any of the radiation-induced tumors. However, dysregulation of several members of the p53 pathway was identified, including MDM2, p21 and BCL2 in 62%, 48% and 29% of cases, respectively.

**Conclusions:** Our data suggest that chromosomal instability does not play a significant role in the pathogenesis of radiation induced thyroid neoplasms. Although p53 aberrations are rare, expression of other members of the p53 pathway is commonly abrogated, suggesting that this pathway plays a role in radiation-induced thyroid cancer pathogenesis.

A

n association between radiation exposure and childhood thyroid neoplasia was first suggested in the early 1950s, with the description of antecedent history of radiation exposure in 10 of 28 children presenting with thyroid cancer to Memorial Hospital in New York City.[1] The most persuasive clinical evidence implicating a causal role for radiation in thyroid tumorigenesis comes from the 1986 Chernobyl nuclear power plant accident.[2] The fallout from the reactor exposed the surrounding population to large quantities of radioiodines, which are incorporated into the thyroid gland after inhalation and/or ingestion. As a result, a more than 100-fold increased incidence of childhood papillary thyroid carcinomas (PTC) has been observed in the population from highly exposed regions including parts of Belarus, Ukraine and The Russian Federation, with the first cases appearing as early as 4 years after exposure.[3] Although the Chernobyl accident has substantiated the causal relationship between radiation exposure and the development of thyroid neoplasms (PTC and follicular thyroid adenomas), the mechanisms underlying tumorigenesis remain ill defined.

*In vitro* evidence suggests that radiation exerts its carcinogenic effect through the induction of genomic damage.[4-6] Accordingly, prior studies focused on identification of genetic aberrations in radiation-induced thyroid cancers in an attempt to define underlying pathogenetic mechanisms.[7-11] A landmark study by Zitzelsberger and colleagues identified a unique recurrent pattern of structural chromosomal alterations in radiation-induced thyroid cancers using conventional cytogenetic karyotyping.[12] In
addition, Richter and colleagues detected an array of genetic abnormalities in Chernobyl-associated PTC including loss of heterozygosity (LOH) events and instability of microsatellite repeats (MSI).[13] The accumulation of DNA damage in radiation induced thyroid cancers appears to be independent of p53 dysfunction, as prior studies demonstrate a low rate of p53 abrogation in these tumors.[14-16] However, no studies have performed an unselected, genome-wide assessment of Chernobyl associated thyroid neoplasia. In addition the p53 signaling pathway, which is involved in the response to radiation induced DNA damage through the regulation of cell cycle control, apoptosis and genomic stability [17], has not been assessed inclusively. We applied comparative genomic hybridization (CGH) [18] to study the molecular-cytogenetic composition of Chernobyl-associated neoplasms. In addition, we assessed the integrity of the p53 pathway including p53, MDM2, p21, Bcl2, cyclin D1 and ki-67 by immunohistochemical analysis of Chernobyl-associated thyroid tumors arrayed on tissue microarrays (TMA)[19] to begin to define the pathogenetic mechanisms of radiation induced thyroid neoplasms.

**Materials and Methods:**

**Case collection and clinicopathological profile:**

The study population included 85 cases of radiation-induced thyroid neoplasia; 27 follicular adenomas and 58 papillary carcinomas. Patients with PTC included 42 females and 16 males. Patients with follicular adenoma included 21 females and 9 males. All patients included were children below the age of 15 that had been exposed to radioactive fallout from the Chernobyl nuclear power plant accident in 1986. Patients with PTC ranged in age between 9 and 15 years with a median of 12 years. Patients with follicular adenoma ranged in age between 2 and 15 years with a median of 13 years. At the time of the Chernobyl accident, 73 of our patients were living in the oblasts of Kiev, Chernigov, Zhytomyr, Sumy, Nikolaev, and Volyn in the Ukraine and 12 patients were living in Bryansk, Russia. All of these patients underwent thyroidectomy between 1995 and 1998. Patients with PTC ranged in age between 1 and 16 years. The clinicopathological and morphological characteristics of our cases have been described previously.[20]

**Comparative genomic hybridization:**

Twenty-eight cases of radiation-induced papillary carcinomas were analyzed by CGH. This cohort included 12 cases from Russia and 16 cases from the Ukraine, based on availability of cancer tissue. Genomic DNA was extracted from paraffin-embedded tumor tissue as described previously.[21] Tumor DNA was labeled by nick translation (Life Technologies, Inc., Rockville, MD) with fluorescein-12-dUTP. Reference DNA was extracted from normal placenta and labeled with Texas red-5-dUTP (New England Nuclear-DuPont, Boston, MA). We have previously validated the use of paraffin embedded tissue derived DNA for CGH in our laboratory.[22] CGH was performed as described.[23, 24] For analysis, 7-10 separate metaphases were captured and processed using the Quantitative Image Processing System (Quips Pathvisyon System; Applied Imaging, Santa Clara, CA). Red, green, and blue fluorescence intensities were analyzed
for all metaphase spreads, normalized to a standard length, and statistically combined to show the red:green signal ratio and 95% confidence intervals for the entire chromosome. DNA copy number changes were detected based on the variance of the red:green ratio profile from the standard of 1. Ratio values of 1.2 and 2.0 were defined as thresholds for gains and amplifications, respectively, and losses were defined as a ratio value of 0.8 or less.

**Tissue micro-array construction and immunohistochemical analysis:**

A tissue micro-array containing 73 radiation-induced tumors from the Ukraine was constructed as described in detail previously.[25] Briefly, two experienced pathologists conducted a critical histologic slide review to confirm the diagnosis and identify representative areas containing over 70% tumor cells in 73 specimens. Corresponding tissue blocks were collected and from the defined areas core biopsies were arrayed in triplicate on a recipient paraffin block using a precision instrument (Beecher Instruments, Silver Spring, MD). Five-μm sections of these tissue array blocks were cut and placed on charged poly-L-lysine-coated slides. The content of individual cores was confirmed by review of hematoxylin and eosin stained slides every ten sections. These sections were subjected to immunohistochemical analysis. Normal thyroid tissue from patients with sporadic papillary carcinomas operated on at Memorial Sloan-Kettering Cancer Center served as baseline controls.

Mouse antihuman monoclonal antibodies to p53 (Ab-2, clone 1801, 1:500; Calbiochem, Cambridge MA), MDM2 (Clone 2A10, 1:500; kindly provided by Dr. A. Levine, Rockefeller University, New York, NY), p21 (Ab-1, clone EA10, 1:100; Calbiochem), cyclin D1 (Ab-3, clone DCS-6, 1:100; Calbiochem), Bcl-2 (clone 124, 1:72; DAKO, Glostrup, Denmark), and Ki-67 (Mib-1, 1:50; Immunotech, Marseille, France) were used for immunohistochemistry as described in detail previously.[26] Tissue loss is a significant factor for tissue array-based analysis, with previously reported rates of tissue damage ranging from 3 to 33%. [25, 27-29] In our analysis, the rate of lost cases attributable to tissue damage was 3-17% for the individual markers. Immunoreactivity was classified as continuous data (undetectable levels or 0% to homogeneous staining or 100%) for all markers. For every marker, the entire tumor tissue of the three core sections was evaluated. A consensus score was obtained between three investigators reading
slides under a multi-headed microscope (VBW, RAG, AO). Cut-off values used in this study were based on previously established cut-off values for well-characterized antibodies used in our laboratory.[26] The cut-off values for tumor cell staining were defined as follows: 1) high Ki-67 proliferative index if >5% tumor nuclei stained; 2) p53 nuclear overexpression if >5% tumor nuclei stained; 3) MDM2 overexpression if >50% tumor nuclei stained; 4) cyclin D1 overexpression if >5% of tumor nuclei stained; 5) Bcl-2 overexpression if >50% of tumor cells demonstrated cytoplasmic staining; 6) p21 overexpression if >10% of tumor nuclei stained. Tumors were then grouped into two categories defined as follows: normal expression (neoplasms below the defined cut-off value of immunoreactivity in normal, benign, and tumor cells) and abnormal expression (normal and neoplastic tissues above the defined cut-off values of immunoreactivity).

Statistical analysis:

Statistical analyses were performed using the JMP4 statistical software package (SAS Institute Inc, Cary, NC.). Qualitative and quantitative differences between malignant and benign thyroid tumors within our series were assessed using the Fisher's exact test and Mann-Whitney $U$-test, respectively. Statistical significance was defined as a two-tailed $p$-value less than or equal to 0.05. Where appropriate, the $p$-value for accepting significance was adjusted for the effect of multiple comparisons by Bonferroni's method.

Results and Discussion:

In this study, we assessed the contribution of chromosomal instability and abroga-

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Normal, matched normal thyroid tissue; RI-A, radiation-induced follicular thyroid adenoma; RI-PTC, radiation-induced papillary thyroid carcinoma
Number of patients in subgroups less than total number in respective group reflects tissue loss during tissue microarray processing
*p-value of comparison between benign tumors vs. malignant tumors (Fischer's exact test)

Table 1. Molecular expression profiles of cell-cycle regulators in thyroid neoplasms (n=85) and normal thyroid tissue (n=33)
tion in the components of the p53 pathway to the pathogenesis of Chernobyl-associated thyroid neoplasms in patients from the Ukraine (Kiev, Chernigov, Zhytomyr, Sumy, Nikolaev, and Volyn) and The Russian Federation (Bryansk) presenting during the period between 1995 and 1998. The etiological impact of the Chernobyl accident on tumorigenesis in these children is supported by the virtual absence of PTC and follicular adenomas in children from these regions that were born at least 2 years after the Chernobyl accident.[30] Application of CGH to the genetic analysis of 28 cases detected chromosomal abnormalities in 2 out of 28 cases (7%). Both of these cases had 2 chromosomal abnormalities each, which are detailed in Figure 1. One case exhibited losses of chromosomal regions 6q12-15 and 16q22-24 and another case had losses of 13q21-31 and Xq21-26. Because the aberrations were non-recurrent, we cannot rule out the possibility that they represent random genetic events. However, it is of note that Zitzelsberger and colleagues detected recurrent deletions of 13q to be associated with radiation-induced thyroid carcinogenesis both in vitro as well as in vivo.[12, 31] In addition, Bauer and colleagues previously reported deletions of chromosomes 6q and 13q in a case of PTC with a history of external beam radiation.[32] Taking into account that these chromosomal abnormalities are rarely present in non-radiation associated PTC,[33, 34] it is possible that these regions may harbor tumor suppressor genes involved in radiation-induced carcinogenesis. However, it needs to be taken into account that accurate comparison of radiation-induced PTC to sporadic PTC would require a matched-pair analysis, a difficult undertaking given the rarity of sporadic PTC in children. Nonetheless, although the carcinogenic effect of radiation is mediated through the induction of genomic instability, it appears from our data that Chernobyl-associated PTC from the Ukraine and Russia do not exhibit a higher frequency of DNA copy number alterations relative to sporadic PTC from Europe and the USA.[22, 32-35]

Our observations sharply contrast with previous findings suggesting that radiation induced PTC commonly contain chromosomal alterations.[7, 12, 13] Zitzelsberger and
colleagues recently detected multiple structural chromosomal alterations in a panel of 56 Chernobyl associated childhood thyroid tumors from the Gomel region of Belarus.[12] In addition, Richter and colleagues detected multiple LOH and MSI events in Belarusian cases.[13] The difference with our data may exist for several different reasons. Firstly, it is possible that the lower rate of chromosomal alterations detected in this study reflects differences in radiation doses received by patients living in the Ukraine and Russian Federation (intermediate dose) and those received by patients living in Belarus (high dose) at the time of the accident.[3, 36] Since induction of chromosomal changes is linearly associated with radiation dose,[7, 12] the higher dose received by Belarusian children may account for the more complex karyotypes detected in Belarusian PTC. Alternatively, it is possible that the degree of chromosomal instability characterizing post-Chernobyl papillary carcinomas is influenced by the latency period between the Chernobyl accident and the time of clinical presentation. For example, the population studied by Zitzelsberger and colleagues includes cases appearing in the years between 1993 and 1996 whereas our study includes cases operated on in the years between 1995 and 1998. Taking into account that chromosomal instability is strongly associated with biologic potential in cancers from various sources including sporadic thyroid cancer,[22, 37] it is possible that early occurring, more aggressive post-Chernobyl cases are characterized by higher rates of chromosomal instability relative to those occurring after longer latency periods. This hypothesis is supported by findings from Lohrer and colleagues reporting an association between the length of the latency period and degree of microsatellite instability in PTC from Belarus.[38] Several other factors may account for the difference in detection rate of chromosomal instability between studies including differences in resolution between the various molecular-cytogenetic techniques that were used (LOH, CGH, conventional cytogenetic karyotyping, microsatellite instability, FISH).[39, 40]

In addition to the presence of chromosomal instability, we assessed the integrity of the p53 pathway in Chernobyl-associated neoplasia. The p53 gene has been shown to play a pivotal role in cellular protection against malignant transformation through its abilities to control the cell cycle, induce apoptosis and safeguard genomic stability.[17] In case of genomic damage, the p53 protein may arrest the cell cycle to allow DNA repair or induce apoptosis through a cascade of downstream factors.[17] Accordingly, the critical role of p53 is evident from the fact that it is mutated in a large fraction of human malignancies.[41] Previous studies have shown that p53 mutations are rare in Chernobyl-associated thyroid neoplasia.[14-16] However, these studies have not taken into account the role of other components of the p53 pathway in Chernobyl-associated neoplasia. We characterized the expression patterns of p53 and some of its most important cellular effectors including Murine double minute 2 (MDM2),[42] p21,[43] cyclin D1,[44] Bcl-2,[45] and Ki-6746 in a morphologically well-characterized cohort including 73 radiation-induced thyroid tumors (follicular adenomas and PTC) arrayed using tissue microarray technology. Our findings are detailed in Table I and Figure II.
Molecular Profiles of Radiation-Induced Thyroid Cancer

Our findings corroborate prior studies showing an absence of \( p53 \) aberrations in Chernobyl-associated thyroid tumors.[14-16] However, our data suggest that dysregulation of cell cycle control and apoptosis in Chernobyl associated thyroid cells may be achieved through abrogation of effectors of \( p53 \) pathway, for example, we found that a large fraction (62%) of cases featured over expression of MDM2, a \( p53 \) binding protein that shuttles \( p53 \) into degradative pathways.[42] Amplification and overexpression of MDM2 has been shown as an important mechanism to dysregulate \( p53 \) in tumors with wildtype \( p53 \).[47] Importantly, we did not find significant differences between malignant tumors (63%) and benign adenomas (62%) indicating that MDM2 dysregulation may be an early event in the development of thyroid neoplasia.

In addition to a high rate of MDM2 dysregulation, we detected \( p21 \) abnormality in 48% of Chernobyl associated PTC. \( p21 \) is a key downstream \( p53 \) target that is transcriptionally regulated by \( p53 \).[43] In case of genomic damage, \( p53 \) mediates cell cycle arrest through \( p21 \).[43] However, several lines of evidence support the presence of a \( p53 \) independent pathway for \( p21 \) activation with divergent biological and clinical activities.[48] Interestingly, we found that expression of \( p21 \) was rare in benign adenomas and very common in PTC \((P=0.008)\), suggesting that \( p21 \) dysregulation may contribute to the malignant phenotype of the radiation-induced thyroid tumors. No significant correlation was present between MDM2 expression and \( p21 \) expression. Our findings suggest that \( p21 \) is dysregulated independent of \( p53 \) or MDM2 in radiation-induced thyroid neoplasia.

Cyclin-D1 is an important antagonist of \( p53 \) functional activity.[49] Cyclin D1 regulates the G1 checkpoint of the cell cycle, which is the point of arrest by \( p53 \). Cyclin D1 amplification and subsequent overexpression is associated with cell cycle dysregulation in a wide variety of tumors including sporadic thyroid cancers.[50] Our data show that cyclin D1 dysregulation is rare in Chernobyl associated thyroid tumors, suggesting that cell cycle dysregulation occurs independently.

In addition to regulation of the cell cycle, \( p53 \) mediates regulation of apoptosis in part through the BAX/Bcl-2 apoptotic pathway.[45, 51] In this balanced system, Bcl-2 inhibits apoptosis and promotes tumorigenesis while BAX acts as a pro-apoptotic factor. Based on previous studies, a cut-off value of for Bcl-2 expression was defined as > 50% tumor cells being positive for Bcl-2.[26] As described previously, normal thyroid tissue was characterized by strong Bcl-2 expression, contrary to observations made in other tumor types.[52] This paradoxical finding could hypothetically be explained by the simultaneously strong expression of pro- and anti-apoptotic components of the apoptotic pathway in normal thyroid tissue. In tumors, the balance of these counteracting molecules may be shifted in favor of inhibiting apoptosis. This hypothesis is supported by recent in vivo studies in mice using gain and loss of function models of BAX and Bcl-2,[57] but needs to be confirmed in the context of the human thyroid. Indeed, in Chernobyl associated neoplastic thyroid tissues, we found downregulation of Bcl-2 in 19% of adenomas and 29% of papillary carcinomas. The difference between adenomas
and carcinomas was not significant. BCL-2 under-expression did not correlate with expression of any other factors that we investigated.

Ki-67 is a nuclear factor associated with cellular proliferation.[46] We chose to investigate the expression of Ki-67 since it is affected by a variety of pathways involved in cell cycle regulation. Moreover, Ki-67 expression has been shown to correlate with clinical behavior in a variety of human tumors including thyroid carcinomas.[52-54] Sporadic PTC and follicular adenomas rarely manifest Ki-67 overexpression consistent with their low biologic potential. Interestingly, our data indicate an absence of Ki-67 expression in radiation-induced thyroid cancers. The absence of Ki-67 in our cases suggests that radiation-induced tumors have an equally low-grade nature as their sporadic counterparts, suggesting excellent overall survival in this patient population.

In conclusion, our data suggest that unbalanced chromosomal abnormalities are rare in Chernobyl associated thyroid tumors from Ukraine and Russia. In addition, our findings corroborate the paucity of p53 alterations in Chernobyl-associated thyroid neoplasms. Dysregulation of cell cycle control, apoptosis and genomic stability may be achieved through abrogation of other effectors in the p53 pathway, including MDM2, p21 and Bcl2. Additional investigations of the molecular-genetic composition of Chernobyl associated cancers are required to further define the involved oncogenetic pathways.

References:

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