

RET EXPRESSION AND ITS CORRELATION WITH CLINICOPATHOLOGIC DATA IN PAPILLARY THYROID CARCINOMA

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SUMMARY – The purpose of this study was to analyze the possible prognostic value of RET mutation in papillary thyroid carcinoma and its incidence in the past few decades in our population, due to the increasing incidence of papillary thyroid carcinoma. The present study included 180 patients operated for papillary thyroid carcinoma. The clinical and histopathologic characteristics were analyzed. Paraffin sections of the selected histologic slides were cut again and immunohistochemically stained by the Clone 3F8 P (HIER) from Novocastra (Vision Bio Systems Europe, Newcastle upon Tyne, UK) monoclonal antibody to RET oncoprotein. Univariate analysis indicated sex ($p=0.01$), histologic subtype ($p=0.075$) and capsular invasion ($p=0.010$) to be statistically significant predictors of lymph node metastases, whereas age ($p=0.796$), tumor size ($p=0.556$) and intraglandular dissemination ($p=0.131$) showed no such correlation. The presence of RET mutation ($p=0.704$) was not a statistically significant predictor of the tumor metastasizing potential. RET mutation ($p=0.500$) showed no statistically significant correlation with papillary thyroid carcinoma classified into prognostic groups according to clinicopathologic features either. RET mutation was detected in 30% of 180 papillary thyroid carcinomas. This is the first large study demonstrating that RET mutation incidence in papillary thyroid carcinoma in Croatian population is consistent with the classic distribution of sporadic cases, despite the increased prevalence of papillary thyroid carcinoma in the past few decades.

Key words: *Mutation; Thyroid cancer, papillary; Chernobyl nuclear accident; Lymphatic metastasis; Radiation*

Introduction

Rearrangement of the tyrosine kinase receptor gene (RET gene named RET/PTC) is the most common structural genetic alteration which, however,

shows great geographical variability ranging from 0 to 80% in different studies¹. In addition, BRAF point mutation, TRK gene rearrangement and RAS mutation have been found in papillary carcinoma²⁻⁴.

RET proto-oncogene has been identified in hereditary MEN2 syndrome, and is involved in sporadic medullary and papillary thyroid carcinoma (PTC) and Hirschsprung's disease⁵. RET proto-oncogene is located on the long arm of chromosome 10. Genetically activated RET rearrangements are implied in the

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pathogenesis of PTC⁶. Variation in the prevalence of RET activation is believed to be attributable to different methods and sensitivity of the techniques used, and to environmental factors, ionizing radiation exposure in particular¹.

There is no RET immunoreactivity in normal thyroid tissue⁷. The mean frequency of RET mutation in sporadic papillary carcinoma is 20%-30% in adults, rising up to 45%-50% in pediatric and young patients, and being highest (50%-80%) in patients with a history of accidental or therapeutic radiation exposure⁸⁻¹⁰. Accordingly, it appears quite reasonable to believe that the pathomorphologically identical papillary carcinomas must have followed different routes of carcinogenesis characterized by different clinical manifestation. In the last decades, there was a significant increase in the number of PTC, so one of the possibilities was the influence of radiation like that due to Chernobyl nuclear power plant disaster¹¹. Knowing that RET mutation has been proven to be a good marker for its influence, we decided to analyze this relationship and to evaluate if there is increment in the RET mutation incidence following the rising PTC incidence⁹.

Patients and Methods

Patients

The present study included 180 PTC patients operated between January 1, 1999 and December 31, 2001 at ENT Department, Split University Hospital Centre in Split. Clinical data were collected from patient medical records from Department of Nuclear Medicine files and histology findings from Department of Pathology and Cytology database. Clinical and pathologic data were analyzed and classified into two groups according to study criteria (age, sex, tumor size, histologic subtype, intraglandular dissemination, extrathyroid dissemination, and metastases): group 1 with favorable prognosis (low risk) included patients aged <45, female, tumor size up to 2 cm, papillary and follicular subtype limited to the thyroid, no intraglandular dissemination, and absence of metastases; and group 2 with poor prognosis (high risk) included all those that did not meet all group 1 criteria, i.e. age >45, male, tumor size exceeding 2 cm, all subtypes except for papillary and follicular ones, capsular invasion, intraglandular dissemination, and metastases to lymph

nodes. At the time of the Chernobyl accident, 33% and 66% of patients were aged <30 and >30 years, respectively.

Methods

Tissue for histologic analysis was obtained from operative material, fixed in 4% buffered formalin for 24 h, paraffin embedded, cut into 3- to 5- μ m sections and stained by the standard hemalaun eosin method (HE). Paraffin sections of the selected histologic preparations were cut again and immunohistochemically stained. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) inside a microwave pressure cooker. Slides were immediately placed into a bath of tap water. Sections were washed in TBS buffer for 1x5 minutes, then placed in diluted normal serum for 10 minutes. Then the slides were incubated with primary antibody. The Clone 3F8 P (HIER) from Novocastra (Vision Bio Systems Europe, Newcastle upon Tyne, UK) monoclonal antibody to RET oncoprotein was used, at 1:40 dilution. Then the slides were washed in TBS buffer for 2x5 minutes, incubated in biotinylated secondary antibody, washed in TBS buffer for 2x5 minutes again, incubated in ABC reagent, washed in TBS buffer for 2x5 once more, incubated in DAB and washed thoroughly in running tap water. Slides were counterstained in hematoxylin, dehydrated and mounted.

The presence of RET/PTC was determined by the presence of diffuse cytoplasmic staining on the normal measuring scale (Fig. 1). The absence of staining indi-

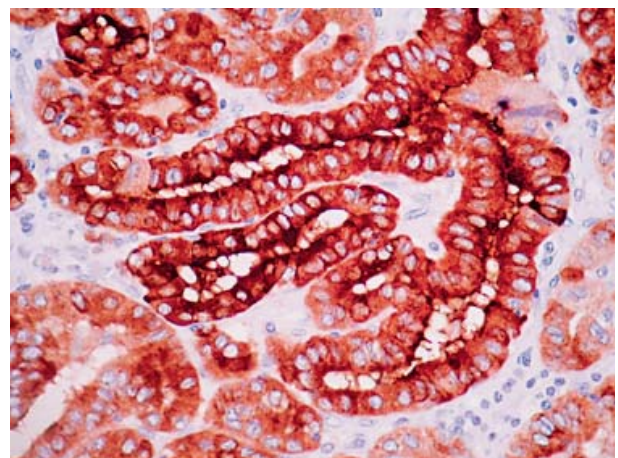


Fig. 1. Diffuse cytoplasmic staining indicates the presence of RET/PTC.

cated absence of the study mutation. Small intestine ganglial cells were used as positive control¹².

Statistics

Descriptive statistics was employed for distribution of data obtained in the patient sample, whereas Pearson's χ^2 -test, Fisher exact test and multivariate analysis of binary logistic regression were used on correlation of the results obtained. The level of statistical significance was set at $p < 0.05$.

Results

In the study population, patients aged >45 as an unfavorable prognostic factor prevailed, with a 2:1 older to younger group ratio. Sex distribution of the study population showed as many as 79.4% of female patients, yielding a 1:3.8 male to female ratio.

According to the new TNM classification, T1 tumor has a borderline size of 2 cm, which was also adopted in the present study. Using this size criterion, carcinomas of up to 2 cm in size predominated ($n=130$; 72.2%). Based on the defined prognostic features of histologic subtypes and trying to facilitate analysis, papillary carcinomas were classified into two categories of favorable and unfavorable subtypes.

Papillary and follicular subtypes were classified into favorable category and accounted for as many as 146 (81.1%) cases, whereas unfavorable category included high cellular, diffusely sclerosing and oncocytic subtypes, found in 34 (18.9%) cases. Intraglandular dissemination (multifocality) was observed in 44.4% of study carcinomas. Current state-of-the-art indicates capsular invasion as a definitely poor prognostic factor. This lesion was recorded in 28 (15.6%) and lymph node metastases in 38 (21.1%) carcinomas. Although distant metastases from PTC are extremely rare, sporadic cases have been reported from similar large studies. In our study, however, distant metastases were not recorded at all. Analysis of immunohistochemical staining for RET mutation revealed its presence in 57 (31.7%) patients (Table 1).

Analyzing particular characteristics of papillary carcinoma in our patients, we assessed the impact of each clinical and histologic feature on the presence of metastases. Patient age had no effect on the prevalence of metastases, as indicated by the results of χ^2 -test ($p=0.796$) and Fisher exact test ($p=0.849$) (Table 2).

Table 1. Frequency of RET mutation in papillary carcinoma

| RET | n | % |
|---------|-----|------|
| Absent | 123 | 68.3 |
| Present | 57 | 31.7 |
| Total | 180 | 100 |

Table 2. Lymph node metastasis in relation to clinical and histopathologic parameters

| Variable | Lymph node metastasis | | |
|--------------------------------|-----------------------|-------------|----------------|
| | Yes n (%) | No n (%) | Total n (%) |
| Age (yrs) ^a | | | |
| <45 | 48 (33.8) | 12 (31.6) | 60 (33.3) |
| >45 | 94 (66.2) | 26 (68.4) | 120 (66.7) |
| Sex ^b | | | |
| female | 25 (65.8) | 118 (83.1) | 143 (79.4) |
| male | 13 (34.2) | 24 (16.9) | 37 (20.6) |
| Tumor size ^c | | | |
| <2 cm | 26 (68.4) | 104 (73.2) | 130 (72.2) |
| >2 cm | 12 (31.6) | 38 (26.8) | 50 (27.8) |
| Subtype ^d | | | |
| favorable | 27 (71.1) | 119 (83.8) | 146 (81.1) |
| unfavorable | 11 (28.9) | 23 (16.2) | 34 (18.9) |
| Multifocality ^e | | | |
| solitary | 17 (44.7) | 83 (58.5) | 100 (55.6) |
| dissemination | 21 (55.3) | 59 (41.5) | 80 (44.4) |
| Capsular invasion ^f | | | |
| present | 11 (28.9) | 17 (12.0) | 28 (15.6) |
| absent | 27 (71.1) | 125 (88.0) | 152 (84.4) |
| RET mutation ^g | | | |
| present | 13 (34.2) | 44 (31.0) | 57 (69.0) |
| absent | 25 (65.8) | 98 (69.0) | 123 (77.1) |

^a χ^2 -test $p=0.796$; Fisher exact test $p=0.849$; ^b χ^2 -test $p=0.019$; Fisher exact test $p=0.025$; ^c χ^2 -test $p=0.556$; Fisher exact test $p=0.547$; ^d χ^2 -test $p=0.075$; Fisher exact test $p=0.101$; ^e χ^2 -test $p=0.131$; Fisher exact test $p=0.145$; ^f χ^2 -test $p=0.010$; Fisher exact test $p=0.021$; ^g χ^2 -test $p=0.704$; Fisher exact test $p=0.699$

Metastases to lymph nodes were detected in 25 of 143 female patients and 13 of 37 male patients. Results of χ^2 -test ($p=0.019$) and Fisher exact test ($p=0.025$) revealed the prevalence of metastases to be statistically significantly higher in male patients (Table 2).

Considering the borderline tumor size of 2 cm, the ratio of non-metastasizing to metastasizing tumors was 104:26 in the <2 cm group and 38:12 in the >2 cm group. Results of χ^2 -test ($p=0.556$) and Fisher exact test ($p=0.547$) showed that tumor size had no statisti-

Table 3. Immunoreactivity to RET mutation in low- and high-risk patient groups

| RET | Good prognosis group | Poor prognosis group | Total |
|---------|----------------------|-----------------------|----------------------|
| Absent | 13 10.6% 61.9% | 110 89.4% 69.2% | 123 100% 68.3% |
| Present | 8 14.0% 38.1% | 49 86.0% 30.8% | 57 100% 31.7% |
| Total | 21 11.7% 100% | 159 88.3% 100% | 180 100% 100% |

χ^2 -test p=0.500; Fisher exact test p=0.618

Table 4. Multivariate analysis of lymph node metastasis predictors in thyroid papillary carcinoma

| Variable | Significance | Odds ratio |
|------------------------------|--------------|------------|
| Age (yrs) | 0.384 | 0.660 |
| Sex (male/female) | 0.058 | 2.409 |
| Tumor size | 0.366 | 0.648 |
| Tumor subtype | 0.569 | 1.343 |
| Intraglandular dissemination | 0.301 | 1.556 |
| Capsular invasion | 0.083 | 2.519 |
| RET | 0.828 | 1.102 |

cally significant impact on the occurrence of metastases (Table 2).

Division of PTCs into favorable and unfavorable subtypes revealed the presence of metastasizing potential in 27 of 146 and 11 of 34 patients, respectively. The χ^2 -test (p=0.075) and Fisher exact test (p=0.101) yielded a statistically significantly greater rate of metastases in the group of unfavorable subtypes (Table 2).

There was no statistically significant difference in the prevalence of metastases according to the presence of intraglandular dissemination (multifocality) (χ^2 -test: p=0.131; Fisher exact test: p=0.145) (Table 2).

Metastases were detected in 27 of 152 cases of PTC without capsular invasion and in as many as 11 of 28 cases with capsular invasion, yielding a statistically significant between-group difference (χ^2 -test: p=0.010; Fisher exact test: p=0.021). Accordingly, the prevalence of metastases was influenced by the presence of capsular invasion (Table 2).

There was no statistically significant difference in the prevalence of metastases according to the presence or absence of RET mutation as the mechanism of carcinogenesis (χ^2 -test: p=0.704; Fisher exact test: p=0.699) (Table 2).

According to the set criteria for good and poor prognosis, 21 PTC cases met all the criteria for good prognosis, whereas 159 high-risk cases were associated with poor prognosis.

There was no statistically significant difference in the frequency of RET mutation as the mechanism of carcinogenesis between the two prognostic groups of PTC patients (χ^2 -test: p=0.500; Fisher exact test: p=0.618) (Table 3).

Univariate analysis was followed by multivariate analysis of the PTC characteristics according to the occurrence of metastases.

Binary logistic regression identified two variables that statistically significantly influenced the occurrence of metastases, i.e. male sex associated with a 2.409 risk of metastases recorded in female patients (p=0.058) and capsular invasion associated with a 2.519 risk of metastases found in patients free from invasion (p=0.083) (Table 4).

Discussion

Immunohistochemical methods of staining that enable detection of specific markers of particular cell types of normal and tumor tissue have found application in the diagnosis of thyroid carcinomas. It is known that no RET immunoreactivity is found in normal thyroid tissue⁷. In the pathogenesis of PTC, RET activation is frequently induced by radiation, mostly at younger age but also in adults¹¹⁻¹³. RET mutation accounts for 20%-30% of sporadic papillary carcinomas in adults, 45%-50% in children and adolescents, and 50%-80% in patients with a history of accidental or therapeutic radiation exposure⁸⁻¹⁰. In our study population, RET mutation was detected in 57 (31.7%) patients (Table 1), which is consistent with most studies on sporadic papillary carcinoma. These results confirmed our hypothesis that radiation exposure like the one caused by Chernobyl disaster did not contribute to the increased incidence of papillary carcinoma in the population.

Investigating the incidence of PTC during the last four decades, Jung *et al.* found that the proportion of

RET/PTC rearrangements decreased, so the increased incidence of PTC was not due to environmental or therapeutic radiation¹⁵.

Analysis of correlation between the classic parameters of PTC and their metastasizing potential revealed sex, tumor size, carcinoma variant and capsular invasion to influence the occurrence of metastases, whereas age and multifocality had no statistically significant impact on the tumor metastasizing potential (Table 2). These results are consistent with the current state-of-the-art on the role of these pathologic and clinical characteristics in papillary carcinoma², thus our study group represented an appropriate model for additional analyses.

Analyzing the correlation between RET mutation and classic prognostic parameters in papillary carcinoma, some authors demonstrated RET mutation to be present at a higher frequency in small, slow progressing and less aggressive tumors, and conclude that it may serve as a favorable marker in these tumors^{16,17}. This is supported by Sugg *et al.*, who identified this mutation mostly in occult microcarcinomas¹⁸. An opposite concept has been proposed by Miki *et al.*, based on the finding of higher RET expression in association with extrathyroid tumor invasion¹⁹.

Sassolas *et al.* report similar results in a population of young adults, where the presence of RET/PTC (1 and 3) was associated with histologic and clinical short-term aggressiveness, but no such relationship was found in children and adolescents²⁰.

In the present study, RET association with papillary carcinomas divided into prognostic categories (Table 3) according to the classic clinical and pathologic parameters was investigated. Categorization into prognostic groups by use of very strict criteria for favorable prognosis (low-risk group): age <45, female sex, tumor size up to 2 cm, papillary or follicular variant confined to the thyroid, no intraglandular dissemination, and no metastases; and poor prognosis (high-risk group): those not meeting all the former group criteria, showed as many as 88.3% of study patients to belong to the high-risk group, thus highlighting the possible factors for poor prognosis. However, there was no statistically significant difference in the prevalence of RET mutation between these prognostic groups of PTC patients (χ^2 -test: $p=0.500$; Fisher exact test: $p=0.618$) (Table 3). This finding is highly comparable with the report by Shin *et al.* on the association of

RET expression with the clinicopathologic parameters of age, sex, tumor size, intraglandular dissemination, lymph node metastases and extrathyroid invasion¹⁴.

The researchers investigating the post-Chernobyl prevalence of papillary carcinoma in Ukraine and in young population exposed to therapeutic irradiation in childhood found a high percentage of RET mutation associated with an increased rate of lymph node metastases^{7,9,21}. In contrast, Collins *et al.* found the association of young age and lymph node metastases to be more pronounced in the group of patients without a history of radiation exposure. In their study, age difference between patients with and without lymph node involvement was only recorded in the group of patients without a history of radiation exposure, thus the increased prevalence of metastases could be directly related to younger age⁷. The association between RET mutation and metastasizing potential has been investigated in many studies. Jhiang *et al.* suggest that RET might also be associated with distant metastases in sporadic cases²², while Mayr *et al.* postulate it to correlate with early dissemination to lymph nodes²³, which is less probable in case of distant metastases.

More recent results reveal connection of a higher rate of RAS mutation with the less aggressive nature of encapsulated follicular variant of PTC, which differs from the more aggressive infiltrative follicular variant, whereas opposite holds for the BRAF mutation pattern²⁴.

In our study, there was no association of RET mutation with lymph node metastases, thus there was no statistically significant difference in the prevalence of metastases according to the presence or absence of RET mutation as the mechanism of carcinogenesis (χ^2 -test: $p=0.704$; Fisher exact test: $p=0.699$) (Table 2). The more so, RET mutation was the first parameter excluded by the stepwise method, implying its lowest possible impact (Table 4).

Considering obvious variation in the results reported from different studies including ours, it appears quite reasonable to presume that some other factors may also influence the prognostic significance of RET mutation. Also, contribution to the increased incidence of papillary carcinoma in our population is probably due to different factors such as better diagnostic procedures and more sophisticated medical technology.

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Sažetak

UČESTALOST RET MUTACIJE U PAPILARNOM KARCINOMU ŠTITNJAJE
I KORELACIJA S KLINIČKO-PATOLOŠKIM KARAKTERISTIKAMA

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Cilj ovoga rada bio je ispitati moguće prognostičko značenje RET mutacije u papilarnom karcinomu štitnjače i učestalost mutacije u odnosu na porast učestalosti papilarnog karcinoma štitnjače u posljednjih nekoliko desetljeća. U istraživanje je bilo uključeno 180 bolesnika operiranih zbog papilarnog karcinoma štitnjače. Analizirane su kliničke i patohistološke osobitosti. Histološki rezovi iz parafinskih blokova odabranih uzoraka imunohistokemijski su obojani monoklonskim protutijelom na RET onkoprotein Clone 3F8 P (HIER) proizvođača Novocastra (Vision Bio Systems Europe, Newcastle upon Tyne, UK). Univarijatnom analizom utvrđena je statistički značajna povezanost spola ($p=0,01$), histološkog podtipa ($p=0,075$) i kapsularne invazije ($p=0,010$) kao prediktora pojave metastaza u limfne čvorove vrata, dok takva povezanost nije zabilježena za dob ($p=0,796$), veličinu tumora ($p=0,556$) i intraglandularnu diseminaciju tumora ($p=0,131$). Prisutnost RET mutacije nije bila statistički značajan prediktor metastatskog potencijala tumora ($p=0,704$). Također, RET mutacija nije bila statistički značajno povezana s prognostičkim skupinama papilarnog karcinoma koje su sastavljene na temelju kliničko-patoloških osobitosti ($p=0,500$). RET mutacija bila je prisutna u 30% od 180 papilarnih karcinoma štitnjače. Ovo je prvo veće istraživanje kojim je dokazano da je RET mutacija u papilarnom karcinomu štitnjače u Hrvatskoj u skladu s učestalošću pojave spontane mutacije, iako se bilježi porast učestalosti papilarnog karcinoma štitnjače tijekom posljednjih desetljeća.

Ključne riječi: *Mutacija; Tireoidni karcinom, papilarni; Černobil, nuklearna nesreća; Limfatične metastaze; Radijacija*