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Significance of Biologic Aggressiveness and Proliferating Activity in Papillary Thyroid Carcinoma

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Abstract. Papillary thyroid carcinoma is a frequent thyroid cancer. Many factors have been reported to be of prognostic importance, but the significance of biologic factors suggesting aggressiveness and proliferating activity has not been sufficiently documented. Conventional prognostic factors such as age, extrathyroidal invasion, lymph node and distant metastasis, and biologic factors including histologic differentiation, DNA ploidy, S-phase and G_2M-phase fractions, and expression of CD44 variant 6 (CD44-v6) obtained from 131 patients who underwent surgery for papillary thyroid carcinoma at Osaka Police Hospital were analyzed retrospectively. Age was closely related to extrathyroidal invasion, G_2M-phase fraction, and CD44-v6 expression. Extrathyroidal invasion was independently related to age, gender, and lymph node metastasis. The grade of lymph node metastasis was related to extrathyroidal invasion, gender, distant metastasis, and CD44-v6 expression. Distant metastasis was associated with aneuploid tumors. Cause-specific survival was independently related to biologic factors including extrathyroidal invasion, distant metastasis, DNA ploidy and S-phase fraction. These results suggest that biologic factors indicating aggressiveness and proliferating activity are important for papillary thyroid carcinoma.

Papillary thyroid carcinoma is a frequent, though relatively indolent, malignant thyroid tumor. This carcinoma with local invasion, locoregional lymph node metastasis, or distant metastasis at diagnosis is associated with substantial postoperative morbidity and mortality. Many factors have been reported to be of prognostic importance for patients with papillary thyroid carcinoma [1–5], including age, sex, histologic features, extrathyroidal invasion, lymph node involvement, and distant metastasis [3, 6, 7]. Among these factors, age, extrathyroidal invasion, and distant metastasis at diagnosis are most frequently reported and are the most powerful prognostic factors, although papillary thyroid carcinoma with extrathyroidal invasion and distant metastasis is infrequent [1, 3, 6, 7]. The high-risk group of patients with papillary thyroid carcinoma indicated by these factors are candidates for aggressive treatment approaches based on individual prognostic factors [7, 8].

The prognostic importance of age in patients with thyroid cancer was suggested owing to the greater prevalence of pathologic risk factors in older patients [9]. We have reported that biologic aggressiveness indicated by immunostaining for the P53 protein and DNA ploidy were independent prognostic factors for overall survival of patients with differentiated thyroid cancer [10]. These results suggest that biologic factors may provide new approaches for inspection of postoperative recurrences and the prognosis of papillary thyroid carcinoma, and that they add another point of view regarding conventional prognostic factors. Although age, extrathyroidal invasion, and lymph node and distant metastasis affect the prognosis, the relation of these factors to biologic factors remains incompletely defined. In this investigation, to reevaluate conventional risk factors for recurrence and poor prognosis, including age, extrathyroidal invasion, and lymph node and distant metastasis, we employed several new biologic factors potentially influencing recurrence and the prognosis of papillary thyroid carcinoma. The biologic factors used were histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44 variant 6 (CD44-v6).

Patients and Methods

Patient Profiles

During the period from 1976 to 1995 a total of 131 patients with primary or secondary papillary thyroid carcinoma underwent surgery at the Department of Surgery, Osaka Police Hospital; their records were examined retrospectively. The subjects were 33 men (25%) and 98 women (75%), and their age at diagnosis was 54 ± 16 years (mean ± SD). Altogether 120 patients (92%) had primary papillary thyroid carcinoma and 11 patients (8%) secondary carcinoma. There were 37 patients (29%) with no lymph node metastasis, whereas 93 patients (71%) had varying degrees of lymph node metastasis. A detailed evaluation of lymph node metastasis could not be made for one patient. Distant metastasis was evident in 9 patients (7%) at diagnosis, and 120 patients

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(92%) were free from distant metastasis at diagnosis. Detailed data of distant metastasis could not be obtained for two patients. On July 1, 1997 there were 108 patients alive; 23 patients had died during the follow-up period. Among these 23 patients, 15 died of thyroid cancer and 8 of unrelated causes. The follow-up period was 5.5 ± 4.5 years.

Operative Methods

Our approach to thyroid carcinoma is macroscopically complete resection of the thyroid tumor by lobectomy plus isthmectomy or total thyroidectomy and central cervical plus ipsilateral jugular lymph node dissection when a tumor is limited to one lobe [10, 11]. Eighty-one patients (62%) underwent unilateral lobectomy + isthmectomy, and 45 (34%) underwent total or near-total thyroidectomy. Five patients (4%) had partial resection of the thyroid gland including the tumor. Altogether 111 patients (85%) underwent central neck dissection plus either unilateral or bilateral jugular lymph node dissection, and 20 patients (15%) had berry pick dissection or no dissection.

All patients routinely received thyroid-stimulating hormone (TSH) suppression therapy after surgery. No patients received prophylactic radioiodine.

Histologic Differentiation

All histologic diagnoses were reviewed by one of the authors (M.T.). Papillary thyroid carcinomas were classified as well or poorly differentiated carcinoma according to the criteria of Sakamoto et al. [12]. Briefly, the characteristic histology of a poorly differentiated carcinoma was the presence of a solid, trabecular, or scirrhous pattern [12]. Among the 126 patients so evaluated, the final histologic differentiation was 69 (55%) well-differentiated and 57 (45%) poorly differentiated papillary thyroid carcinomas.

Flow Cytometry

Nuclear suspensions were prepared from paraffin-embedded tissue blocks according to the technique of Hedley et al. [13], with slight modifications. The isolated nuclei were stained with propidium iodide by the method of Rainwater et al. [14]. They were then analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA, USA). At least 10,000 cells were analyzed by flow cytometry. A diploid DNA histogram showed a peak in the diploid region (DNA index = 0.95–1.05), and a tetraploid DNA histogram displayed a prominent peak in the tetraploid region (DNA index = 1.90–2.10). Both these patterns are classified as diploid herein. DNA histograms outside these regions were considered to be aneuploid. In this study, 75 carcinomas (84%) were diploid and 14 carcinomas (16%) aneuploid. Percent values (mean ± SD) of the S and G_2M phases in the cell cycle were 3.30 ± 2.69 (n = 78) and 5.22 ± 3.36 (n = 79), respectively.

Immunohistochemistry

Rabbit polyclonal antibodies against full-length recombinant human P53 protein (CM-1; Novocastra, Newcastle, UK) and paraffin-embedded tissue blocks were used. A conventional avidin–biotin complex method (Dako A/S; Alostrup, Denmark) and a peroxidase–diaminobenzene method were used to stain for the P53 protein as described previously [15]. A total of 59 tumors were stained for the P53 protein; and in this study, if a tumor had a positively stained cancer cell immunohistochemically, the tumor was considered to be positive for the P53 protein. It was negative for 39 tumors (66%) and positive for 20 (34%). In all tumors, fewer than 10% of the tumor cells were positive immunohistochemically for the p53 protein.

For immunostaining of CD44v6, the VFF-18, anti-human CD44-v6 monoclonal antibody (mouse IgG1), which specifically recognizes an epitope encoded by exon CD44-v6, was purchased from Bender MedSystem (Vienna, Austria). Immunostaining of CD44-v6 was performed essentially as described in a previous report [16]. With immunostaining of CD44-v6, the cancer cells stained in whole plasma membrane were considered positive for CD44-v6. When a part of the plasma membrane was stained, cells were considered negative for staining. Because the distribution of stained cancer cells was not always homogeneous, a tumor was considered positive for CD44-v6 in the present investigation when at least 10% of cancer cells were positive for CD44-v6. Among the 90 who were subjected to this test, 62 (69%) had CD44-v6-positive cancers and 28 (31%) had CD44-v6-negative cancers.

Statistical Analysis

The relative relations between conventional prognostic factors and other possible prognostic factors were analyzed by the multiple regression analysis or the logistic regression analysis with the forward stepwise method. Analyzed factors were age, gender, extrathyroidal invasion, lymph node metastasis, distant metastasis, primarity, histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44-v6. The relative importance of various prognostic factors for postoperative survival as identified by multivariate analysis was analyzed with the Cox's proportional hazards model with the forward stepwise method. The possible prognostic factors analyzed included operative methods, dissection of regional lymph nodes, and the above described factors. Assumptions of proportional hazards had been tested. In multivariate analyses, unknown values of expression of CD44-v6, flow cytometry, and histologic differentiation were replaced by mean values of the other patients because of the limited number of patients. A p value less than 0.05 was considered significant. All statistical analyses were performed using a commercially available personal computer program, SPSS (SPSS, Chicago, IL, USA).

Results

Relation between Conventional Prognostic Factors and Other Factors

Differentiated thyroid carcinomas in older patients showed a high prevalence of pathologic risk factors including extracapsular extension [9]. In the present investigation, age was closely related to extrathyroidal invasion (absent = 0, present = 1), the G_2M-phase fraction in the cell cycle analysis, and expression of CD44-v6 (negative = 0, positive = 1) (Table 1). Conversely, as shown in Table 2, extrathyroidal invasion was closely related to age, gender (female = 0, male = 1), and lymph node metastasis (absent = 0, present = 1).

Table 1. Independent risk factors for age and the grade of lymph node metastasis.

Risk factor	Coefficient	SE	*p*
Age (adjusted r = 0.944 and $p < 0.0001$)			
Extrathyroidal invasion[a]	11.350	2.540	< 0.0001
G_2M-phase fraction	1.367	0.492	0.0063
CD44-v6 expression[a]	6.204	3.038	0.0432
Grade of lymph node metastasis[b] (adjusted r = 0.8210 and $p < 0.0001$)			
Gender (female = 0, male = 1)	0.5643	0.1976	0.0050
Extrathyroidal invasion[a]	1.0079	0.1729	< 0.0001
Distant metastasis[a]	1.2787	0.3299	0.0002
Histologic differentiation (well = 0, poorly = 1)	−0.3615	0.1723	0.0379
CD44-v6 expression[a]	0.4536	0.2002	0.0252

SE: standard error.

Possible risk factors for age and the grade of lymph node metastasis were analyzed using multiple regression analysis. Analyzed factors were age, gender, extrathyroidal invasion, lymph node and distant metastasis at diagnosis, the primarity, histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44-v6.

[a]Absent = 0; present = 1.

[b]Absent = 0, movable unilateral metastasis = 1, movable bilateral metastasis = 2, fixed cervical lymph node metastasis or mediastinal lymph node metastasis = 3.

Table 2. Independent risk factors for extrathyroidal invasion, lymph node metastasis, and distant metastasis.

Risk factors	Coefficient	SE	*p*	Odds ratio
Extrathyroidal invasion (absent = 0, present = 1)				
Age	0.0534	0.0140	0.0001	1.0548
Gender	0.9532	0.4828	0.0483	2.5940
Lymph node metastasis	1.6463	0.5026	0.0011	5.1876
Lymph node metastasis (absent = 0, present = 1)				
Extrathyroidal invasion	1.7540	0.4795	0.0003	5.7776
G_2M-phase fraction	−0.1780	0.0819	0.0299	0.8370
Distant metastasis at diagnosis (absent = 0, present = 1)				
Primarity (primary = 0, secondary = 1)	2.0869	0.8779	0.0175	8.0595
DNA ploidy (diploid = 0, aneuploid = 1)	2.0714	0.7886	0.0086	7.9361

Possible risk factors for extrathyroidal invasion, lymph node metastasis, and distant metastasis at diagnosis were analyzed using logistic regression analysis. Analyzed factors were age, gender, extrathyroidal invasion, lymph node and distant metastasis, primarity, histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44-v6.

Lymph node metastasis (absent = 0, present = 1) showed a close relation with extrathyroidal invasion and the G_2M-phase fraction (Table 2). Expression of CD44-v6 did not show any significant relation to the presence of lymph node metastasis in these settings. However, as shown in figure 1, metastatic cancer cells in lymph nodes expressed CD44-v6 more intensely and broadly than those at the primary site. Furthermore, most metastatic lesions in lymph nodes expressed CD44-v6 regardless of CD44-v6 expression in the primary site (Table 3). Thus similar risk factors were reanalyzed regarding the grade of lymph node metastasis. As shown in Table 1, the grade of lymphatic metastasis significantly correlated with extrathyroidal invasion, gender, distant metastasis (absent = 0, present = 1), histologic differentiation (well = 0, poorly = 1), and expression of CD44-v6.

Finally, the risk factors for distant metastasis at diagnosis were analyzed (Table 2). The presence of distant metastasis was found to be significantly related to the primary status of the tumors (primary = 0, secondary = 1) and DNA ploidy (diploid = 0, aneuploid = 1).

Risk Factors for Recurrence and Prognostic Factors

Risk factors for newly developed locoregional recurrences were examined using the Cox's proportional hazard model with the forward stepwise method. The analysis indicated that distant metastasis ($p < 0.0001$) and DNA ploidy ($p = 0.0025$) were independent risk factors for locoregional failures, with relative risks of 17.220 and 4.530, respectively. For newly developed distant metastatic recurrences, distant metastasis ($p < 0.0001$), gender ($p = 0.0184$), and DNA ploidy ($p = 0.0255$) were extracted with relative risks of 520.72, 5.646, and 5.159, respectively.

The 5- and 10-year overall survival rates of patients with papillary thyroid carcinoma in this study were nearly 85% and 80%, respectively (Fig. 2). The independent prognostic factors analyzed are shown in Table 4. For overall survival, the age, DNA ploidy, and distant metastasis are indicated as independent prognostic factors. On the other hand, extrathyroidal invasion, distant metastasis, DNA ploidy, and S-phase fraction were independent prognostic factors for cause-specific survival, and age was no longer an independent prognostic factor in this setting.

Discussion

Possible biologic indices reported as prognostic factors include expression of oncogenes and suppressor genes, such as the *ras* family, c-*erbB,* P53 protein, and *ret* oncogenes [10, 17]. Oncogenes and suppressor genes are important in the oncogenesis and prognosis of thyroid cancer [17]. Others have noted that cell cycle analysis is a useful tool for determining the prognosis [18, 19]. We have shown that both P53-positive staining and DNA ploidy are independent prognostic factors for overall survival of patients with differentiated thyroid carcinoma, and that examination of biologic factors may provide new information regarding postoperative recurrences and prognosis [10]. In the present investigation, we evaluated several conventional prognostic factors and biologic factors in papillary thyroid carcinoma. Biologic factors include histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44-v6. Histologic differentiation and DNA content are thought partially to represent the proliferative activity of the cancer. Cell cycle analysis has been reported to be a good indicator of proliferative activity [18–20]. Values for S- and G_2M-phase fractions are the percent of cancer cells in S and G_2 plus M phases of the cell cycle, respectively, for which the values indicate cells undergoing mitosis and the proliferative activity of the cancer. The P53 protein is a multifunctional protein and regulates the cell cycle [21]. Positive staining by P53 immunohistochemistry was observed mainly in tumors that had molecular alterations of the *p53* gene and is associated with overexpression or functional inactivation of the protein [22]. Reports have suggested that CD44 is a cell adhesion molecule and a homing receptor of activated lymphocytes to the lymph node, and that

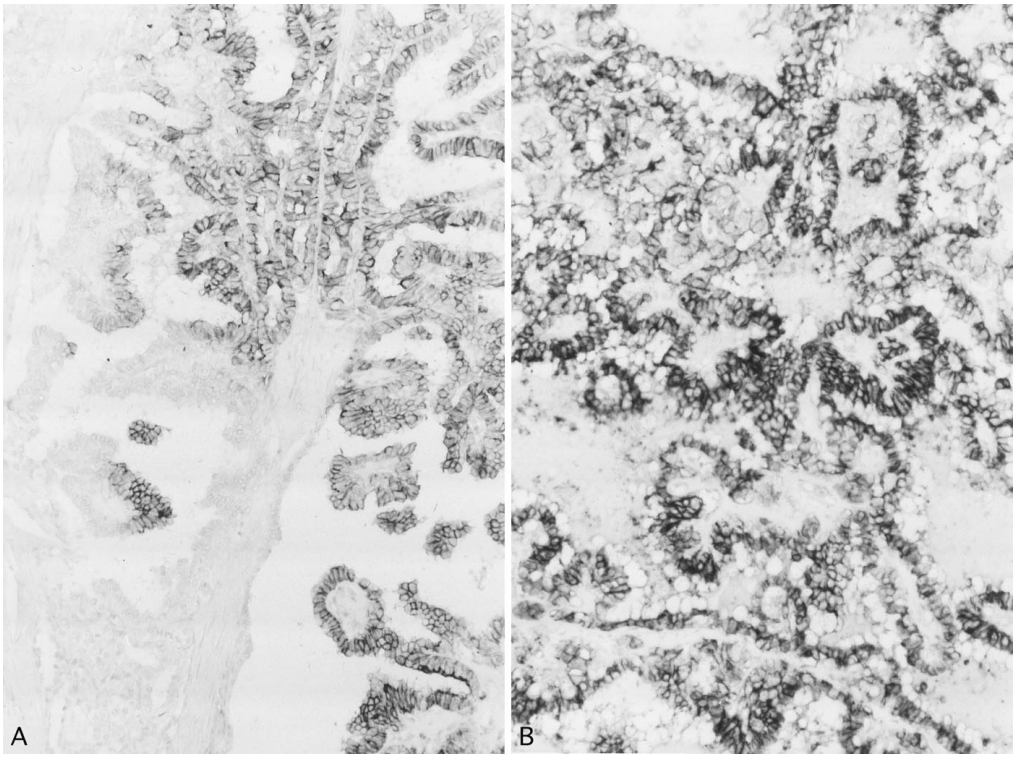

Fig. 1. Typical representatives of immunohistochemistory of CD44 variants 6 (CD44-v6) in papillary thyroid carcinoma. **A.** Immunostaining with CD44-v6 for well-differentiated papillary thyroid carcinoma. **B.** Metastatic lesion in the cervical lymph nodes obtained from the same patient. The intensity of immunostaining and the CD44-v6-stained area were increased in the metastatic lesions.

Table 3. Relation between CD44-v6 expression in the primary site and metastatic lymph nodes.

CD44-v6 expression in the primary site	CD44-v6 expression in metastatic lymph nodes	
	Negative	Positive
Negative	0	12
Positive	2	23

The numbers of patients are shown.

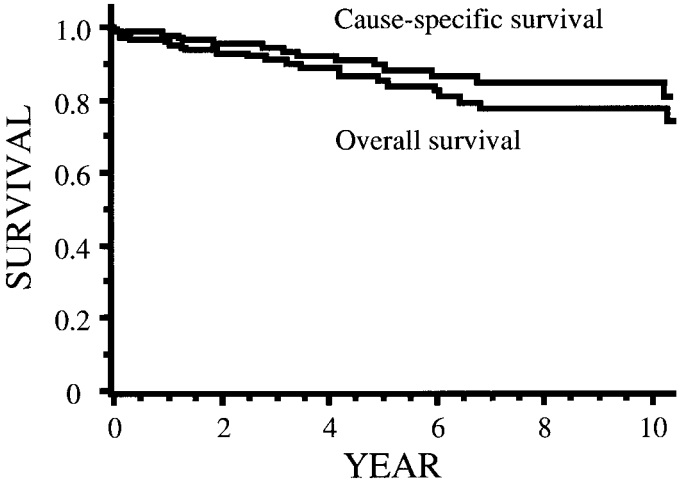

Fig. 2. Postoperative overall and cause-specific survivals of patients with papillary thyroid carcinoma. The Kaplan-Meier method for postoperative overall survival shows that 5- and 10-year survival rates were 84.8 ± 3.8% and 78.0 ± 4.4%, respectively. The 5- and 10-year cause-specific survivals were 89.2 ± 3.4% and 83.8 ± 4.4%, respectively.

expression of its variant, CD44-v6, plays an essential role in tumor progression and metastasizing behavior [23–25]. Thus these biologic factors used in the present investigation are thought to represent proliferative activity and the biologic aggressiveness of the cancer.

It was reported that thyroid carcinoma in elderly patients frequently showed vascular invasion and extracapsular extension, and the prognostic importance of age was suggested owing to the greater prevalence of pathologic risk factors [9]. In this report, age was also suggested in close relation with extrathyroidal invasion. Furthermore, the G_2M-phase fraction (suggesting proliferative activity) and CD44-v6 (indicating proliferative and metastasizing activity) were also related to age. These results suggested that patients of older age frequently encountered biologically aggressive thyroid cancer, which may make age prognostic. Age, distant metastatic site, and extrathyroidal invasion were reported to be significant prognostic factors for patients bearing a papillary thyroid carcinoma with distant metastasis [26]. In this report, distant metastasis was shown to be partly regulated by DNA ploidy, suggesting biologic aggressiveness of the cancer. The differences between the two reports may be partly explained by the different clinical settings for the analyses. Another important prognostic factor, extrathyroidal invasion, was predominant in and was independently related to older age, male gender, and the presence of lymph node metastasis (Table 2).

Papillary thyroid carcinoma preferentially metastasizes to regional lymph nodes, and lymph node metastasis is usually considered to have second-line prognostic importance compared to age, extrathyroidal invasion, and distant metastasis. The presence of lymph node metastasis is commonly thought to increase postop-

Table 4. Independent prognostic factors for overall and cause-specific survival.

Prognostic factor	Coefficient	SE	*p*	Relative risk
Overall survival[a]				
Age	0.087	0.020	< 0.0001	1.091
DNA ploidy	2.332	0.497	< 0.0001	10.302
Distant metastasis	1.467	0.633	0.0204	4.337
Cause-specific survival[b]				
DNA ploidy	2.139	0.589	0.0003	8.490
Distant metastasis	2.416	0.711	0.0007	11.195
Extrathyroidal invasion	2.265	0.787	0.0040	9.633
S-phase fraction	0.240	0.101	0.0172	1.271

[a]The other factors, including gender, extrathyroidal invasion, lymph node metastasis, operative method, lymph node dissection, histologic differentiation, S-phase and G_2M-phase fractions, and expression of CD44-v6, were not significant.

[b]The other factors, including age, gender, lymph node metastasis, operative method, lymph node dissection, histologic differentiation, G_2M-phase fraction, and expression of CD44-v6, were not significant.

erative morbidity [6]. Reports suggested that expression of CD44 variants may play an important role in lymphatic metastasis of several cancers [23–25], and CD44-v6 was reported to be expressed in papillary thyroid carcinoma [16, 27]. In the present investigation, the grade of lymph node metastasis, but not the presence of lymphatic metastasis, was found to be related to expression of CD44-v6. This possibility was confirmed by the fact that cancer cells metastasizing in lymph nodes expressed CD44-v6 more frequently and intensely (Table 3, Fig. 1). These results suggest that CD44-v6 expressed in papillary carcinoma may have a role in lymphatic metastasis.

Several scoring systems have been introduced for differentiated thyroid carcinoma [3, 4, 6, 7], many of which include age, extrathyroidal invasion, and distant metastasis, as described above [3, 4, 6, 7]. This report, which includes several biologic factors in the analysis, indicated that age, DNA ploidy, and distant metastasis were of prognostic importance for overall survival; and that distant metastasis, extrathyroidal invasion, DNA ploidy, and S-phase fraction were prognostic factors for cause-specific survival. Age was replaced by extrathyroidal invasion and S-phase fraction for predicting cause-specific survival. These results suggest that cancer-related death closely relates to biologic behavior and the proliferating activity of the cancer.

Conclusions

The significance of biologic factors, including histologic differentiation, DNA ploidy, S- and G_2M-phase fractions, and expression of CD44-v6, was evaluated in relation to conventional prognostic factors, such as age, extrathyroidal invasion, lymph node metastasis, and distant metastasis, and with prognosis. Age was closely related to extrathyroidal invasion, cell cycle, and CD44-v6 expression. The grade of lymph node metastasis was relative to CD44-v6 expression, and distant metastasis was associated with aneuploid tumors. Cause-specific survival was independently related to biologic factors, including extrathyroidal invasion, distant metastasis, DNA ploidy, and S-phase fraction. These results suggest that biologic factors indicating an aggressive nature of cancer cells and proliferating activity are important for the prognosis of patients with papillary thyroid carcinoma.

Résumé

Le cancer papillaire est un des plus fréquents de la thyroïde. De nombreux facteurs pronostiques ont été rapportés. Cependant, parmi eux, les facteurs biologiques, suggérant une agressivité particulière et une activité de prolifération, n'ont pas encore été suffisamment documentés. Les facteurs pronostiques classiques, comprenant l'âge, l'invasion extrathyroïdienne, l'atteinte ganglionnaire et l'existence de métastases à distance et des facteurs biologiques comme la différenciation histologique, la ploïdie AND, les fractions de phase-S et de phase G2M ainsi que l'expression de la protéine CD44 variante 6 (CD44-v6) ont été analysés de façon rétrospective à partir de 131 dossiers de patients opérés d'un cancer papillaire de la thyroïde à l'Hôpital de la Police d'Osaka. On a trouvé une corrélation étroite entre l'âge et l'invasion extrathyroïdienne, la fraction de phase G2M ainsi que l'expression de la protéine CD44v6. L'invasion extrathyroïdienne n'était en rapport ni avec l'âge, ni le sexe, ou les métastases ganglionnaires. L'importance de métastases ganglionnaires était en parallèle avec l'invasion extrathyroïdienne, le sexe, les métastases à distance, et l'expression de CD44-v6. Les métastases à distance corrélaient avec les tumeurs aneuploïdes. La survie était en rapport avec des facteurs biologiques comprenant l'invasion extrathyroïdienne, les métastases à distance, la ploïdie AND et la fraction phase-S. Ces résultats suggèrent qu'il importe de prende en considération les facteurs biologiques indicatifs de l'agressivité et de l'activité prolifération en cas de cancer papillaire de la thyroïde.

Resumen

El carcinoma papilar de la glándula tiroides es un tipo muy frecuente de cáncer. Aunque se han descrito diversos factores sobresalientes de pronóstico, no se ha documentado suficientemente la importancia de los factores biológicos que sugieren virulencia y actividad proliferative. En un grupo de 133 pacientes sometidos a cirugía por carcinoma papilar de tiroides en el Hospital de la Policıá de Osaka se realizó un análisis retrospectivo de factores convencionales de pronóstico tales como edad, invasión extratiroidea, metástasis ganglionares y metástasis distances, y de factores biológicos como diferenciación histológica, ploidia de DNA, fracciones de fase-S y de fase-G2M y expresión de la variante 6 de CD44 (CD44-v6). La edad apareció íntimamente relacionada con invasión extratiroidea, fracción de fase G2M y expresión de CD44-v6. La invasión extratiroidea apareció relacionada en forma independiente con la edad, el sexo y las metástasis ganglionares. El grado de metástasis ganglionares se correlacionó con invasión extratiroidea, metástasis distantes y expresión de CD44-v6. Las metástasis distances se correlacionaron con los tumores aneuploides. La supervivencia por caso específico se vio relacionada en forma independiente con los factores biológicos, incluso, invasión extratiroidea, metástasis distantes, ploidia de DNA y tracción de fase-S. Tales resultados apuntan a que los factores biológicos que sugieren virulencia y actividad proliferativa son de importancia en el pronóstico del carcinoma papilar de tiroides.

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