의학석사 학위논문

Analysis of Clinical Prognostic Significance of Cancer Stem Cell Markers in Patients with Papillary Thyroid Carcinoma

갑상선 유두암 환자에서 암줄기세포 표지자 발현에 따른 임상 예후 분석

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Abstract

Analysis of Clinical Prognostic Significance of Cancer Stem Cell Markers in Patients with Papillary Thyroid Carcinoma

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Introduction: Recently, the cancer stem cells (CSCs) model was new era of excitement in thyroid cancer research, which indicates that a small subset of CSCs existed in tumor cells may contribute to the initiation, progression and recurrence of metastatic disease, as well as therapy resistance. The aim of this study was to evaluated presence of CD44, CD24 and CD133 tumor cells in papillary thyroid carcinoma (PTC) as marker of aggressiveness and poor prognosis.

Methods: All patients with PTC, who underwent successful surgical resections from January 2003 to December 2012 in single tertiary hospital, were included in

this study. Tissue arrays were made with 454 primary tumor tissues. Immunohistochemistry(IHC) of 3 cancer stem cell markers (CD24, CD44 and CD133) were applied to corresponding tissue arrays. IHC was graded by semiquantative histologic scoring considering the extent and intensity of the staining. IHC results were correlated with clinicopathological characteristics and with recurrence free survival.

Results: In total 500 patients, 46 cases were recurred during 70 month median follow-up period. In univariate log rank test of Kaplan Meier survival model, sex (p = 0.010), age (p = 0.005), cN1b (p = 0.000), pN1 > 5 (p = 0.000), pTumor size > 2 cm (p = 0.000), extrathyroidal extension (p = 0.000), CD24 (-) (p = 0.000) were prognostic factors for RFS. CSCs marker combinations such as CD44 (+)/CD24 (-), CD24 (-)/CD133 (+), CD44 (+)/CD24 (-)/CD133 (+) also showed statistically significant in Log Rank test.

Conclusion: Expression of CSC markers CD44 (+), CD24 (-), and CD133 (+) in tissue samples of PTC correlates with RFS. In particular, the combination of CD44 (+) and CD24 (-) showed a statistically significant relationship with RFS and a strong correlation with gross extrathyroidal extension.

Keywords: Papillary thyroid carcinoma, Cancer stem cell, Prognosis, Immunohistochemistry, CD24, CD44, CD133

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LIST OF ABBREVIATIONS

PTC : papillary thyroid carcinoma

CSCs : cancer stem cells

TMA : tissue microarray

IHC : immunohistochemistry

CD24 : cluster of differentiation 24

CD44 : cluster of differentiation 44

CD133 : cluster of differentiation 133

Introduction

Thyroid carcinoma is the most common endocrine malignancy, and its incidence has recently increased globally. In Korea, the diagnosis of thyroid carcinoma has also increased rapidly, accounting for 14.2% of all cancers in 2014[1]. The increase of thyroid cancer is mostly due to the increase of papillary thyroid carcinoma (PTC). PTC has a very good prognosis, but even after appropriate treatment, several years to several decades after treatment, 10-20% of patients experience recurrence, and 2-5% of them develop distant metastasis[2]. Since some of the patients who undergo recurrence will eventually die of cancer, the establishment of prognostic factors for recurrence is an important part of the treatment of PTC.

Recently, the cancer stem cells (CSCs) model has ushered in a new era of excitement in thyroid cancer research, which indicates that a small subset of CSCs existed in tumor cells may contribute to the initiation, progression and recurrence of metastatic disease, as well as therapy resistance[3,4]. Characterization of CSCs composition within tumors can provide novel target therapeutic models using this cell specificity, and eventually improve the survival of patients with aggressive thyroid cancer. The development of novel CSCS-targeted strategies for anti-tumor therapy relies on the identification of CSCs specific biomarkers[5]. With advancement of knowledge, the well-accepted cancer stem cell surface markers are CD44, CD24, CD133, CD166, EpCAM, and so forth, in different tumors including breast, lung, pancreas, prostate, colorectal,

renal, and ovarian, while the prognostic value of these markers is still under investigation. CSCs[6].

CD44 is a cell surface glycoprotein that is expressed on lymphocytes, monocytes and granulocytes, and has been recognized as a CSC marker in breast, pancreas, and head and neck cancer[7,8]. CD24 is a small cell surface protein molecule anchored by glycosyl-phosphotidyl-inositol in a wide variety of cancer cells. It is heavily glycosylated and functions in cell-cell and cell-matrix interactions. The inverse relationship between CD24 expression and tumorigenicity has been well documented by Pruszak et al[9]. CD133, also known as prominin-1, is a five-transmembrane domain glycoprotein specifically expressed on the surface of hematopoietic stem and progenitor cells. Previous studies have shown that CD133 is a marker of CSCs in different types of cancer including brain tumors, colon cancer and melanoma[10-12].

In addition, CD44 and CD24 have been used extensively in combination or with other putative markers to isolate CSCs from solid tumors. But the levels of CD44 and CD24 expression show great variation between cell lines even in cells of the same cancer subtype[6]. In Human breast cancer, CD44 (+)/CD24 (-) cells were well recognized as a CSCs, but in ovarian and colorectal cancer it showed ambiguity. CD44 is expressed in almost all normal and cancer cells leading to discrepancy and reflecting the ambiguity regarding functional aspects[13]. In Our previous study[14] also showed high levels of CD44 expression in most cases. So CD44 alone appears to be insufficient for identifying stem cell populations in PTC. Therefore, the combination of CD24 with the proved inverse proportion to the tumorigenicity was proposed as the CSCs marker of PTC.

To the best of our knowledge, there are no studies on the relationship between cancer stem cells and clinical prognosis in PTC. The present study evaluated presence of CD44, CD24 and CD133 tumor cells in PTC as marker of aggressiveness and poor prognosis.

Materials and Methods

Subjects and tissue samples

From July 2003 to December 2012, 500 consecutive PTC patients with a tumor size greater than 1 cm, who underwent successful surgical resection by single experienced surgeon in Seoul National University Bundang Hospital were enrolled in this study. Patients with underwent previous treatment or those who were unable to follow up at least 6 months were excluded. This study was approved by the institutional review board at Seoul National University Bundang Hospital (No. B-1507/306-310). The paraffin blocks of the surgical specimens were examined by two pathologists, Choi JY and Lee KY. Since paraffin blocks of primary tumor tissues were not available in 46 patients, the paraffin blocks of 454 patients were selected to make a tissue microarray (TMA).

Tissue microarray

After review of the 454 tumor tissues, the representative core tissue sections (2 mm in diameter) were taken from paraffin blocks and arranged in new TMA blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). The detailed description of the TMA construction can be found at homepage of the company (http://www.tissue –array.com). After the TMA blocks were constructed, the blocks were sectioned with thickness 4 ㎛ for immunohistochemistry (IHC).

IHC staining

This study evaluated the expressions of three proteins for the tumor tissues; CD44, CD24, and CD133. For IHC, antibodies for the following molecules were used in this study; CD44 (1:600, BOSTER Biological Technology Co., Ltd. CA, USA), CD24 (1:50, abcam, Cambridge, MA, USA), CD133 (1:200, Biorbyt Ltd. Cambridgeshire, United Kingdom). CD44 was rabbit monoclonal antibodies. CD24 was mouse monoclonal antibodies. CD133 was rabbit polyclonal antibodies.

Using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA), slides were stained as follow procedure. Detection was done using the Ventana Chromo Map Kit (Ventana Medical Systems).Sections were deparaffinized using EZ Prep solution. CC1 standard (pH 8.4 buffer contained Tris/Borate/EDTA) was used for antigen retrieval. inhibitor D(3% H_2O_2, Endogenous peroxidase) was blocked for 4 min at 37°C temperature. Slides were incubated with Primary antibodies for 32 min at 37°C, and a secondary antibody of (Omimap anti Mouse) for 20 min at 37°C. Slides were incubated in DAB+ H_2O_2 substrate for 8 min at 37°C followed by Hematoxylin and Bluing reagent counterstain at 37°C. Reaction buffer (pH 7.6 Tris buffer) was used as washing solution.

IHC grades

Immunostaining was evaluated by two independent pathologists who were blind to the experimental design, and the scores were determined semiquantitatively, based on staining intensity and proportion. IHC expression was graded according to the following criteria: 0 (no staining); 1 (weak); 2 (moderate); and 3

(strong)[15]. Points less than equal or one was marked as negative (-), and more than one points were marked as positive (+) for statistical analysis.

Statistical analysis

Pearson's chi-square test was used to analyze the relationship between expressions of IHC and clinicopathological data. The recurrence free survivals (RFS) were investigated with positive and negative expressions of IHC by univariate log rank test in Kaplan Meier survival analysis. Also multivariate cox regression test was performed to identify factor affecting RFS. Results of all statistical tests were defined with the statistical significance at $p < 0.05$ and the marginal significance at $p < 0.10$. SPSS (Version 19.0; SPSS, Inc., Chicago, IL) was used for the statistical analysis.

Results

Clinical characteristics

In total, 500 patients with PTC were included in our study. Relevant demographic, clinical, and pathological data, as well as management and survival of the patients were retrieved and summarized in Table 1. There were more female PTC patients than male patients (384 (76.8 %) vs. 116 (23.2 %)). Their age ranged from 10 to 87 (median 48.0) years. Ninety nine patients (19.8 %) were under 60 years, and remaining 401 patients (80.2 %) were over 60 years old. Ninety nine patients (19.8 %) were suspected to have lateral lymph node metastasis at the time of diagnosis. Histopathologically, 307 patients (61.4 %) had lymph node metastasis less than 5 and 108 patients (21.6 %) had more than 5 lymph node metastasis. According to TNM staging system, there were 118 Patients (23.6 %) in T stage I, 17 (3.4 %) in II, 358 (71.6 %) in III, and 7 in IV (1.4 %). There were 367 patients (73.4 %) with primary tumors less than 2 cm in size and 133 patients (26.6 %) with tumors larger than 2 cm, and 225 (45.0 %) patients showed multifocality. One hundred thirty seven patients (27.4 %) had no extrathyroidal extension, 254 (50.8 %) had microscopic extrathyroidal extension, and 109 (21.8 %) had gross extrathyroidal extension. Total thyroidectomy was performed in most patients (94.6 %). Central lymph node dissection was performed in 309 patients (61.8 %), of which 101 patients (20.2 %) underwent lateral lymph node dissection. The median follow-up period was 70 months and two patients died due to other causes.

Thyroid cancer recurred in 46 patients (9.2 %). Median time to first recurrence was 22 months. The recurrence site was 2 cases of Thyroid remnant or bed, 8 cases of central lymph node area, 34 cases of lateral lymph node area, and 7 cases of distant metastasis.

Correlation of IHC results with clinical data in PTC patients

In most analysis, there was no statistically significant relationship between single stem cell markers alone and clinicopathological data, but Age (p = 0.001), extrathyroidal extension (p = 0.039) and cancer recurrence (p = 0.000) were statistically negative correlation with CD24. (Table 2.)

The correlation of the combined status of CD44 (+), CD24 (-), CD133 (+) with clinicopathological data in PTC patients was further performed. Our results showed that there was a statistically significant relationship between recurrence of cancer in all combinations of CSCs maker except CD44 (+)/CD133 (+). Especially, the combination of CD44 (+)/CD24 (-) also showed a significant relationship with age and gross extrathyroidal extension. (Table 3.)

Recurrence free survival according to the clinical data and IHC

Recurrence free survival (RFS) curves were shown in Figure 1 according to the clinical data and IHC results. In univariate log rank test of Kaplan Meier survival model, sex (p = 0.010), age (p = 0.005), cN1b (p = 0.000), pN1 > 5 (p = 0.000), pTumor size > 2 cm (p = 0.000), extrathyroidal extension (p = 0.000), CD24 (-) (p = 0.000) were prognostic factors for RFS. CSCs marker combinations such as CD44 (+)/CD24 (-), CD24 (-)/CD133 (+), CD44 (+)/CD24 (-)/CD133 (+) also

showed statistically significant in Log Rank test. In multivariate analysis, CD44 (+)/CD24 (-) showed statistically significance with hazard ratio 5.048. (Table 4.)

Disscussion

In general, the results of cancer prognosis and treatment efficacy are ultimately determined according to the survival of the patient, and the criteria for staging are determined based on factors related to survival. However, in the case of differentiated thyroid carcinoma, the progression is very slow and even in the case of recurrence, there is some possibility of cure through reoperation and treatment with radioactive iodine. Even if it is not cured, it can bring about a very long considerable degree of palliation. Therefore, in the case of differentiated thyroid carcinoma, it may be a limitation to determine the prognosis based on survival alone, and considering the recurrence free survival may be a more reasonable approach[16]. In our study, no one died of papillary thyroid cancer during the 70-month median follow-up period, and recurrence rate was 9.2 %, similar to other prognostic studies[17].

CSCs are a small subpopulation of cancer cells characterized by self-renewal, with the capacity to differentiate into several tumor cell types and metastasize[18]. Bonnet and Dick first reported the existence of a CSC population in 1997. They identified a population of leukemic stem cells in human acute myeloid leukemia and demonstrated that CSCs initiated leukemia in NOD/SCID mice[19]. Since then, in 2003, Al-Haj et al has announced a proof of CSCs in breast cancer which was the first to demonstrate CSCs in solid tumors[20]. Subsequently, CSCs have been identified in a number of other solid tumors, including tumors in the brain, prostate, colon, head and neck, lung, melanoma, liver, ovary and pancreas[21].

The thyroid CSCs are can distinguished by the expression of biomarker, and ability to produce thyrospheres in vitro, and ability to make tumors in vivo[22]. Zito et al first attempted to isolate CSCs in 2008, and analyzed the expression of CD133 by flow cytometry in thyroid cancer cell lines[4]. Subsequently, Friedman et al demonstrated that the transplantation of CD133 (+) cells into immunodeficient NOD/SCID mice was sufficient to induce tumor growth in vivo[23]. Our previous study[14] focused on CD44 and CD24, which are CSC markers for some cancers, including breast and colon[24]. Using cancer cell lines (the original TPC1 cell line and its derivatives), we found higher numbers of CD44 (+)/CD24 (−) cells in the more aggressive cell lines (positivity rates being 86% in highly tumorigenic TPC-1Mice cells, >73% in moderately tumorigenic TPC-1SC2 cells, >21% in parental, poorly tumorigenic TPC-1 cells). Subsequently, using dispersed cells from thyroid cancers, we determined that 4–70% cells were CD44 (+)/CD24 (−), the cells formed spheres, CD44 (+)/CD24 (−) but not CD44 (+)/CD24 (+) cells from these spheres were spherogenic, and the cells derived from thyrospheres (at least 1 × 104) formed tumors following orthotopic injection.

However, these results were considered controversial, as Schweppe et al[25] evaluated 40 thyroid cancer cell lines using short tandem repeat and single nucleotide polymorphism array analyses, and reported that a number of the cell lines have been widely used in the thyroid cancer field were not only redundant , but not of thyroid origin. The result that most cell lines of thyroid cancer are cross-contaminated indicates the limitations of using existing cell lines in thyroid cancer stem cell research. Furthermore, the significance of CD133 as a thyroid CSC marker is also controversial. CD133 mRNA was expressed in spheres from

dispersed thyroid cancer cells[26]. CD133 protein was expressed in a FRO cell line but not in a TPC cell line[23] and not in eight cell lines including FRO-mBRAF[27] and cancer-derived thyrospheres/tumors[28]. Therefore, it is necessary to identify thyroid CSCs in human thyroid tissue specimens and to study them. Also IHC evaluation using CSC markers is clearly less common than in vitro and in vivo experiments. So this study was conducted using PTC surgical specimens with tissue microarray and IHC which were developed and generalized.

As far as we know, this study is the first to analyze the relationship between CSCs and clinical prognosis of PTC. In our study, the CSCs markers IHC results were not correlated with the prognostic factors of commonly known in PTC patients, except for the presence of gross extrathyroidal extension. Recently, the AJCC 8th edition for different thyroid cancer downstages a significant number of patients by raising the age cut off from 45 years of age at diagnosis to 55 years of age. This change was confirmed by a good prognosis in young people aged 45 to 55 years in international multi-institutional validation study of 9484 patients[29]. Similarly, in our study, there was no difference in IHC outcome and prognostic analysis at index age 45 years. However, when the index age was increased to 60 years, the differences in CD44 (+)/ CD24 (-), was confirmed.

With Kaplan-Meier analysis we found that there were a significant negative relationship between RFS and CD24 expression. CD44 and CD133 showed positive correlation with RFS, but statistically not significant. In addition, CSCs marker combination analysis including CD44 (+)/CD24 (-) showed statistically significant correlation with RFS. These results are consistent with the findings of Yalan et al., in which the IHC results of CD44 (+)/CD133 (+) in medullary thyroid

carcinoma correlate with survival[5], and that CD44 (+)/ CD24 (-) are associated with prognosis in patients with other carcinomas such as breast[30]. However, the combination of CD24 (-)/CD133 (+) and CD44 (+)/CD24 (-)/CD133 (+) is considered to have a limited clinical application because of the small proportion of cases.

At present, surgery, radiation therapy, chemotherapy and hormonal therapy are used to treat thyroid cancer; however, these treatments often exhibit limited efficacy. Conventional therapies target highly proliferating cells that form the majority of the tumor mass, but they are ineffective against slowly proliferating or quiescent CSCs, which are responsible for drug resistance, metastasis and recurrence[31]. However, the clinical importance of presence of CSCs markers, evaluated with IHC, remains uncertain. Because of their plasticity, whether the cells positive for these markers are actually CSCs is unknown. Even if IHC evaluation precisely reflects cancer stemness, the overall interpretation of such data would still be challenging[30]. But these efforts should be continued because of the ability to identify, isolate and study thyroid CSCs has a number of implications with potential novel therapeutic consequences.

Conclusion

Expression of CSC markers CD44 (+), CD24 (-), and CD133 (+) in tissue samples of PTC correlates with RFS. In particular, the combination of CD44 (+) and CD24 (-) showed a statistically significant relationship with RFS and a strong correlation with gross extrathyroidal extension. Thus we would suggest CD44 (+)/ CD24(-) expression for evaluating prognosis associated to recurrence free survival in PTC.

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국문초록

서론: 암줄기세포 모델은 암세포에 존재하는 작은 부분의 암줄기세포가 전이성 질환의 시작, 진행 및 재발 뿐만 아니라 치료 저항에도 공헌하고 있음이 알려져 최근 들어 감상선암 연구 분야의의 새로운 영역으로 각광 받고 있다. 저자는 이 연구에서 CD 44, CD24, CD133의 발현 정도를 단독 혹은 복합적으로 분석하여 갑상선 유두암에서의 예후인자로서 의미가 있는 지 여부를 밝히고자 한다.

재료 및 방법: 2003년부터 2012년까지 단일 삼차병원에서 갑상선 유두암으로 수술 받은 환자를 대상으로 원발 부위의 암 조직을 수집하였고, 이를 이용하여 파라핀 블록에 조직을 배열하여 보관하였다. 이후 3가지 종양줄기세포 표지자(CD44, CD24, CD133)의 면역조직화학염색을 시행하여 환자의 임상정보와 비교 분석하였다.

결과: 총 500 명의 환자에서 70 개월의 중앙 추적 기간 동안 46명의 갑상선 유두암이 재발되었다. Kaplan Meier 생존 모델의 단변량 로그랭크 테스트에서 성별 (p = 0.010),나이 (p = 0.005), cN1b (p = 0.000), pN1> 5 (p = 0.000), 원발종양크기> 2cm (p = 0.000), 갑상선피막외침범 (p = 0.000), CD24 (-) (p = 0.000)가 재발 없는 생존의 예후 인자였다. CD44 (+) / CD24 (-), CD24 (-) / CD133 (+), CD44 (+) / CD24 (-) / CD133 (+)와 같은 암 줄기세포 표지자 조합에서도 재발 없는 생존과 통계학적으로 유의미한 예후 인자로 분석되었다.

결론: 갑상선 유두암 검체에서 암 줄기세포 표지자인 CD44 (+), CD24(-), 및 CD133 (+)의 발현은 재발 없는 생존과 관련 있다. 특히 CD44 (+)와 CD24 (-)의 조합은 통계적으로 유의한 재발 없는 생존의 예후 인자로 나타났으며 갑

상선피막외침범과 강한 상관 관계를 보였다.

주요어: 갑산선 유두암, 암줄기세포, 예후, 면역조직화학염색

학번: 2013-23489

TABLE 1 Clinical characteristics of study patients (n = 500)

Variable			*N* (%)
Sex			
	Male		116 (23.2)
	Female		384 (76.8)
Age at operation, median (range), years		48.0 (10-87)	
	60<		99 (19.8)
	60≥		401 (80.2)
cN1b			99 (19.8)
pN1			
	≤5		307 (61.4)
	>5		108 (21.6)
pTstage			
	pT1/T2/T3/T4		118/17/358/7 (23.6/3.4/71.6/1.4)
Pathologic tumor size, median (range), cm		1.4 (0.3-7.0)	
	≤2		367 (73.4)
	>2		133 (26.6)
Multifocality			225 (45.0)
Extrathyroidal extension			
	No		137 (27.4)
	Microscopic		254 (50.8)
	Macroscopic		109 (21.8)
Surgery			
	Lobectomy/total thyroidectomy		27/473 (5.4/94.6)
	CND/CND plus LND		309/101 (61.8/20.2)
First relapse			
	Thyroid remnant or bed		2 (0.4)
	Central compartment LN		8 (1.6)
	Lateral compartment LN		34 (6.8)

	Distant site	7 (1.4)
	Median time to first relapse (range), months	22 (2-101)
Follow-up information		
	Median follow-up (range), months	70 (6-141)
	Death (index cancer/other causes)	0/2 (0/0.4)

CND central compartment neck dissection, LN lymph node, LND lateral compartment neck dissection, pTNM pathologic tumor-node-metastasis stage (AJCC, 7th ed.)

TABLE 2 Correlation of CD44, CD24, and CD133 with Clinical Data in PTC Patients

	CD44				CD24				CD133			
	Negative	Positive	X^2	ρ	Negative	Positive	X^2	P	Negative	Positive	X^2	ρ
Age(years)												
60<	8	82	0.590	0.574	50	44	11.889	**0.001**	84	8	0.081	1.000
60≥	41	308			125	244			327	35		
Sex												
Male	13	91	0.246	0.597	40	69	0.073	0.822	92	15	3.376	0.087
Female	36	299			135	219			319	28		
Tumor size												
≤2	34	287	0.391	0.608	132	206	0.840	0.389	297	35	1.652	0.277
>2	15	103			43	82			114	8		
cN1b												
No	41	306	0.714	0.461	136	233	0.684	0.407	322	38	2.384	0.165
Yes	8	84			39	55			89	5		
pN1												
≤5	32	235	1.832	0.252	111	169	0.964	0.352	254	23	0.187	0.684
>5	7	92			47	57			93	10		
Multifocality												
No	29	211	0.454	0.545	91	165	1.233	0.289	224	26	0.560	0.521
Yes	20	179			84	123			187	17		
Extrathyroidal extension												
No + Micro	40	302	0.445	0.587	127	233	4.368	**0.039**	319	33	0.017	0.850
Macro	9	88			48	55			92	10		
Recurrance												
No	48	352	3.191	0.105	148	274	15.061	**0.000**	378	37	1.740	0.245
Yes	1	38			27	14			33	6		

TABLE 3 Correlation of CD44(+)/CD24(-), CD44(+)/CD133(+), CD24(-)/CD133(+), and CD44(+)/CD24(-)/CD133(+) with Clinical Data in PTC Patients

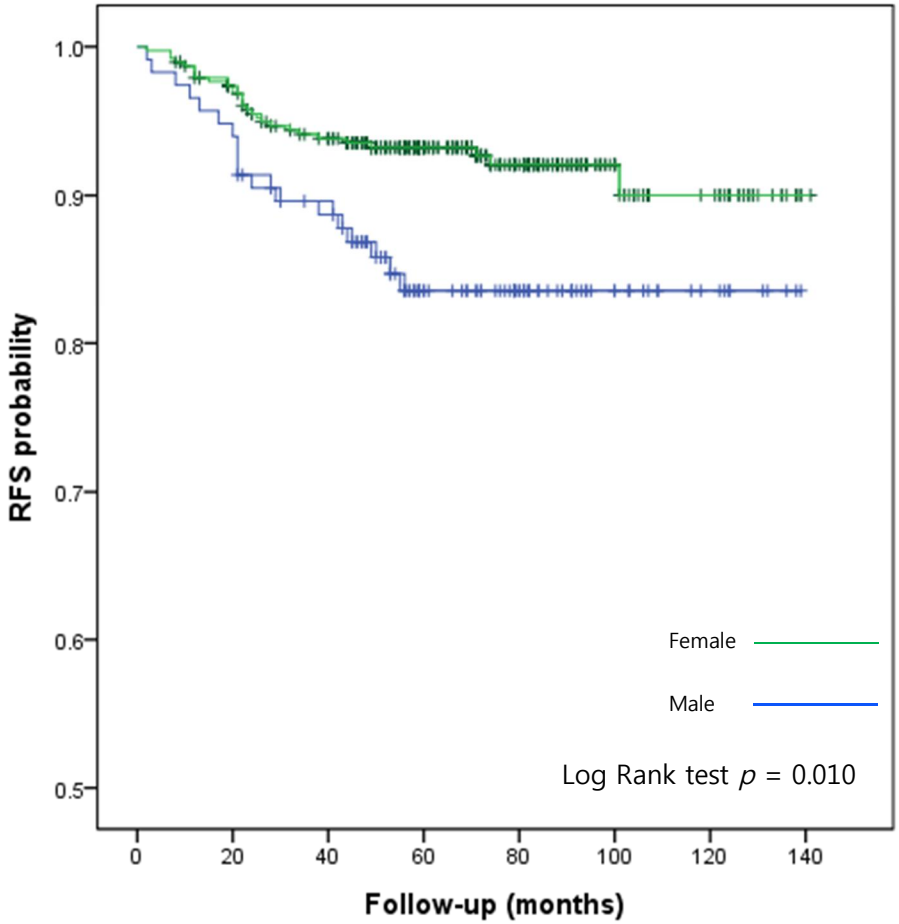
	CD44(+)/CD24(-)				CD44(+)/CD133(+)				CD24(-)/CD133(+)				CD44(+)/CD24(-)/CD133(+)			
	No	Yes	X^2	ρ	No	Yes	X^2	ρ	No	Yes	X^2	ρ	No	Yes	X^2	ρ
Age(years)																
60<	45	44	9.079	**0.004**	82	8	0.000	1.000	87	4	0.244	0.541	85	4	0.885	0.312
60≥	235	112			316	31			349	12			338	9		
Sex																
Male	68	36	0.081	0.815	90	14	3.456	0.076	101	6	1.755	0.228	99	5	1.574	0.202
Female	212	120			308	25			335	10			324	8		
Tumor size																
≤2	203	116	0.176	0.675	288	31	0.915	0.450	318	13	0.544	0.576	309	10	0.096	1.000
>2	77	40			110	8			118	3			114	3		
cN1b																
No	225	120	0.715	0.393	311	34	1.746	0.221	346	13	0.034	0.854	335	10	0.039	0.739
Yes	55	36			87	5			90	3			88	3		
pN1																
≤5	165	99	1.065	0.335	246	19	0.845	0.386	266	9	1.281	0.251	258	6	3.232	0.096
>5	56	43			89	10			97	6			93	6		
Multifocality																
No	161	78	2.275	0.134	216	23	0.317	0.616	238	11	1.251	0.314	230	9	1.124	0.399
Yes	119	78			182	16			198	5			193	4		
Extrathyroidal extension																
No + Micro	227	113	4.351	**0.041**	310	30	0.019	0.842	341	10	2.195	0.216	333	7	4.546	**0.044**
Macro	53	44			88	9			95	6			90	6		
Recurrance																
No	268	129	20.858	**0.000**	365	33	2.199	0.142	402	11	10.767	**0.008**	389	8	14.333	**0.003**
Yes	12	27			33	6			34	5			34	5		

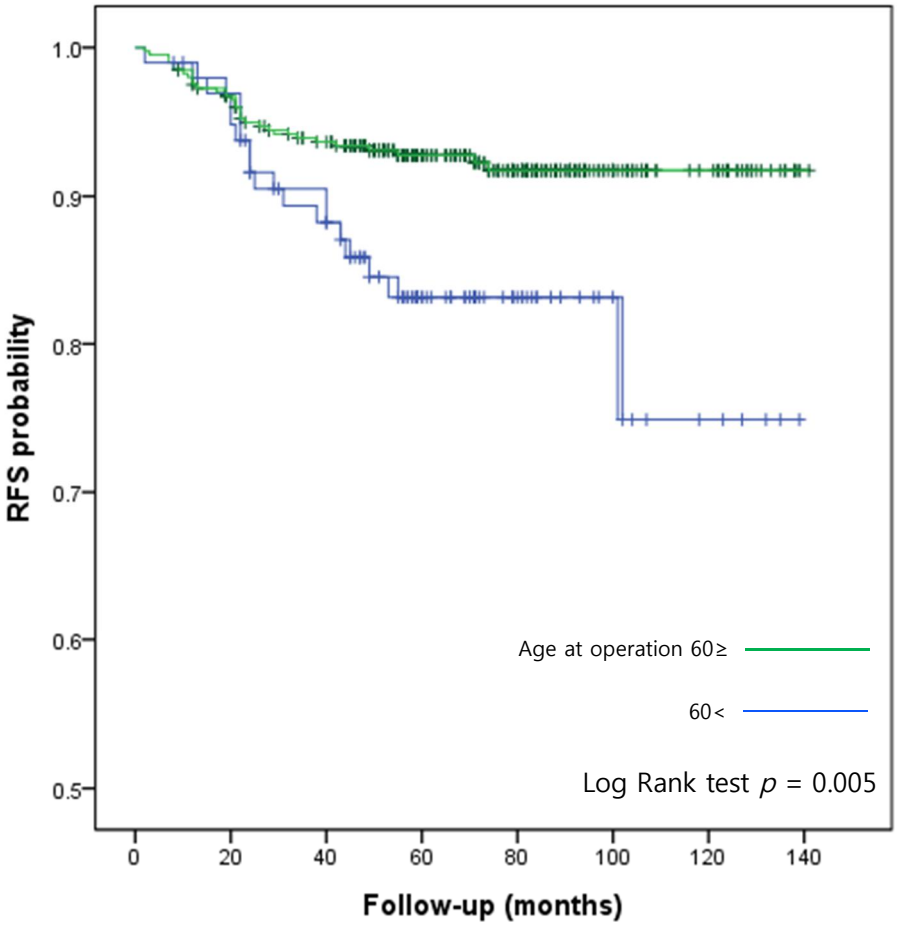
TABLE 4 Multivariate analysis for recurrence-free survival

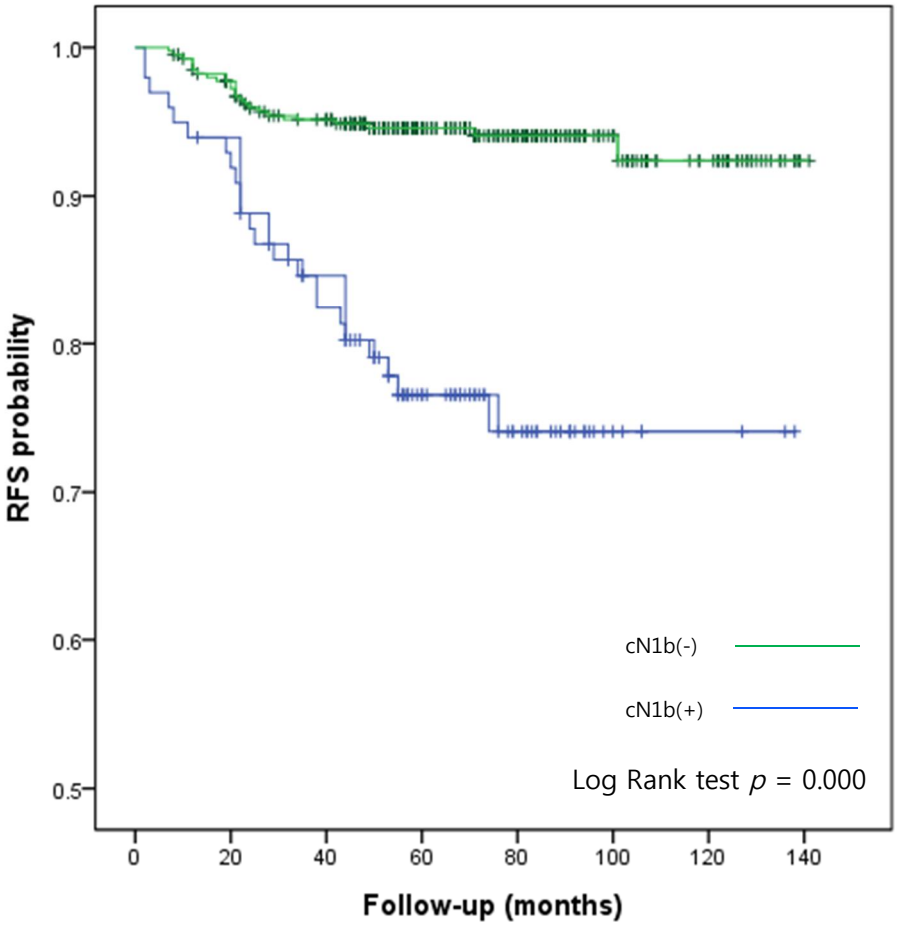
Subjects	Hazard Ratio	95% CI	*P*-value
Age (>60)	1.635	0.759-3.519	0.209
Sex (Male)	2.242	1.116-4.505	**0.023**
Size (>2cm)	2.544	1.123-5.763	**0.025**
cN1b	2.592	0.885-7.594	0.083
pN1 (>5)	2.633	0.882-7.863	0.083
Gross ETE	0.812	0.358-1.842	0.618
CD44(+)/CD24(-)	5.048	2.313-11.016	**0.000**

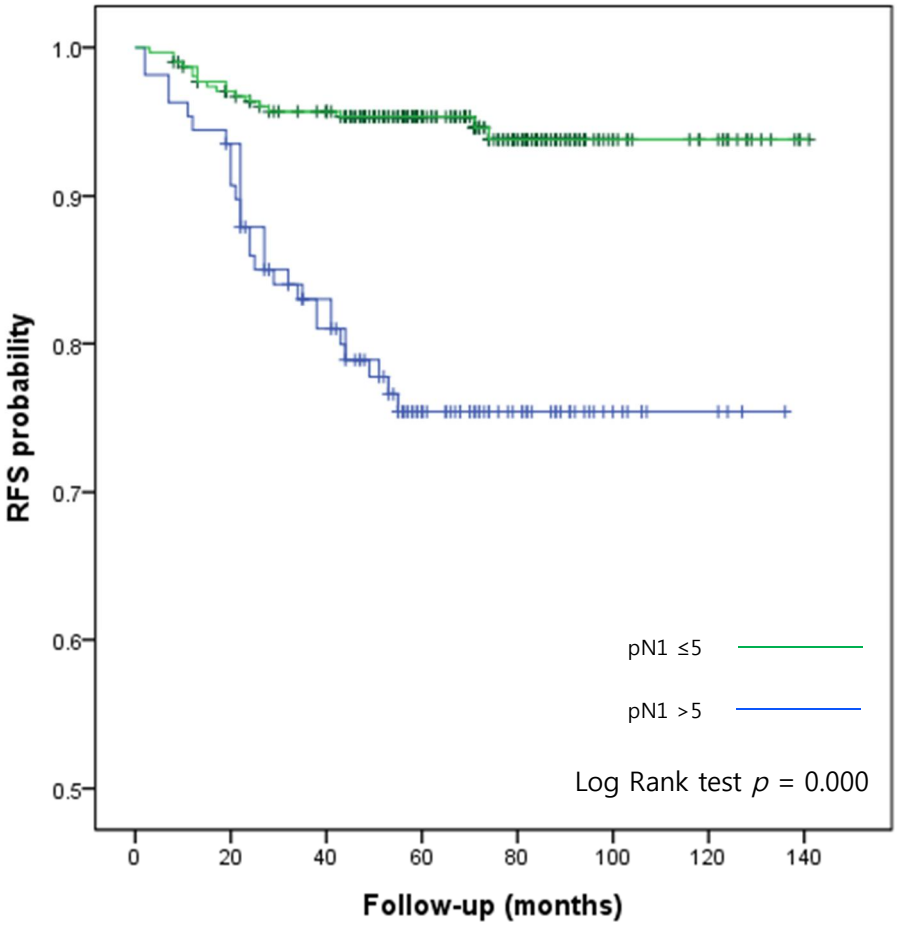
95% CI, 95% Confidence Interval; *P*, significant level for Cox-regression test

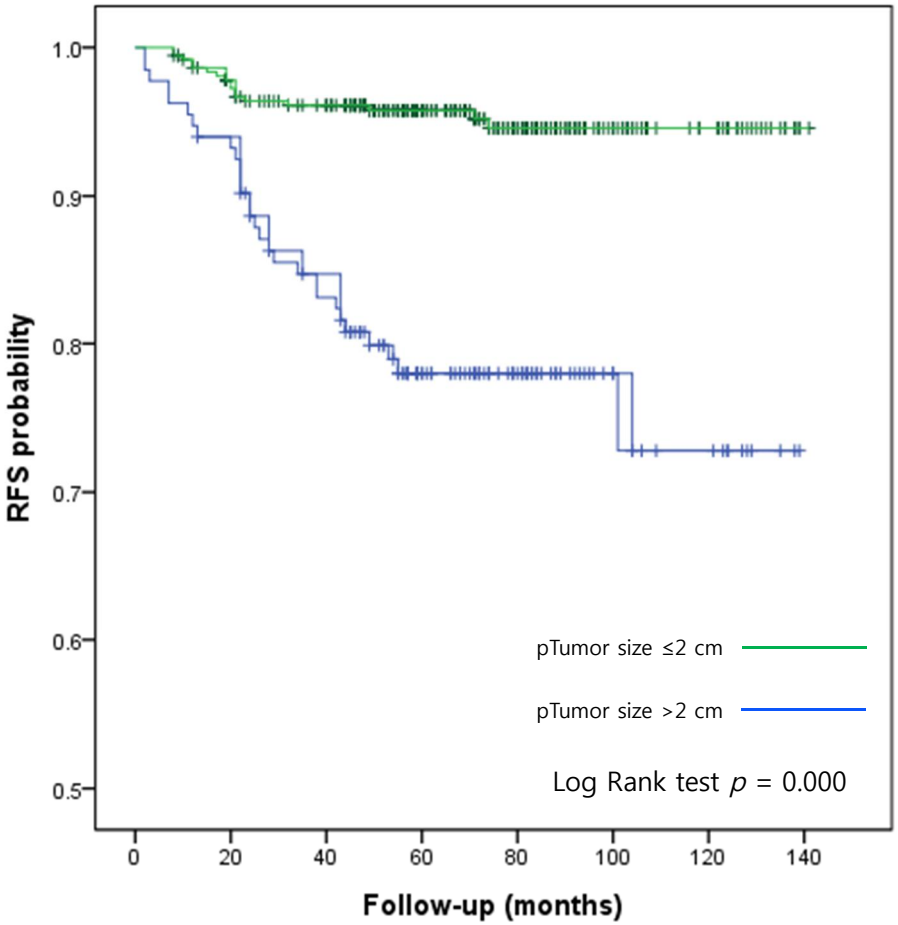
Figure 1 Kaplan-Meier estimates showing recurrence-free survival(RFS) according to the clinical data and immunohistochemistry

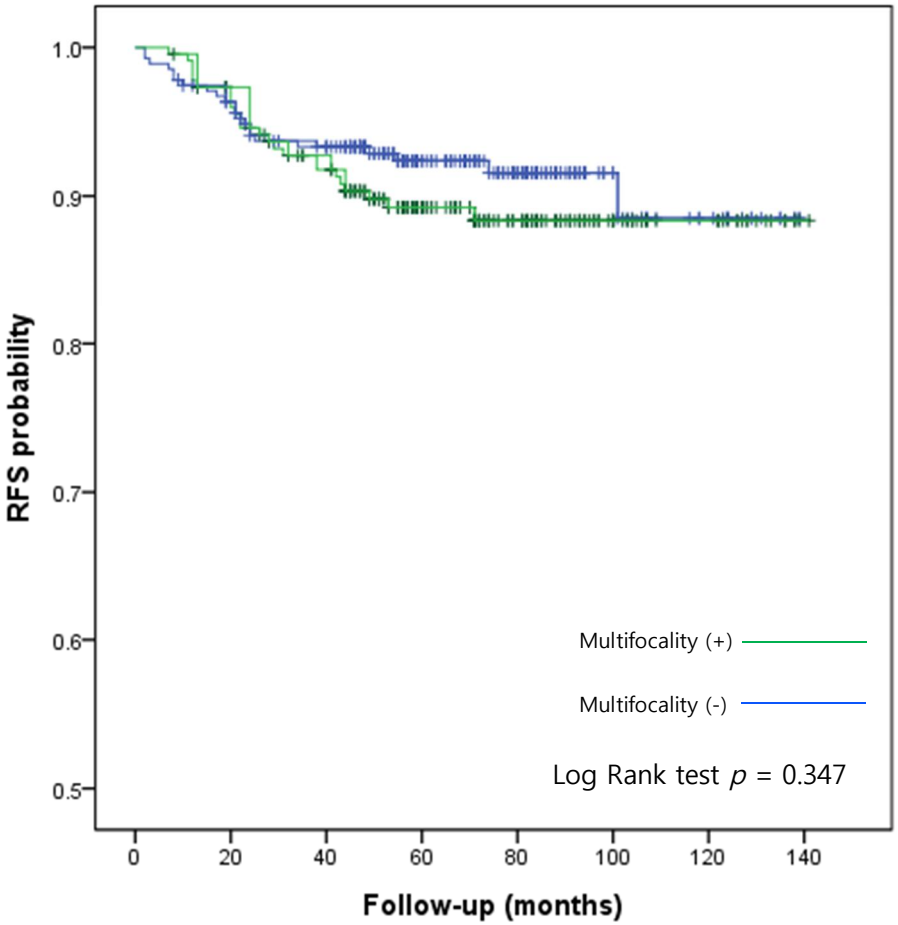


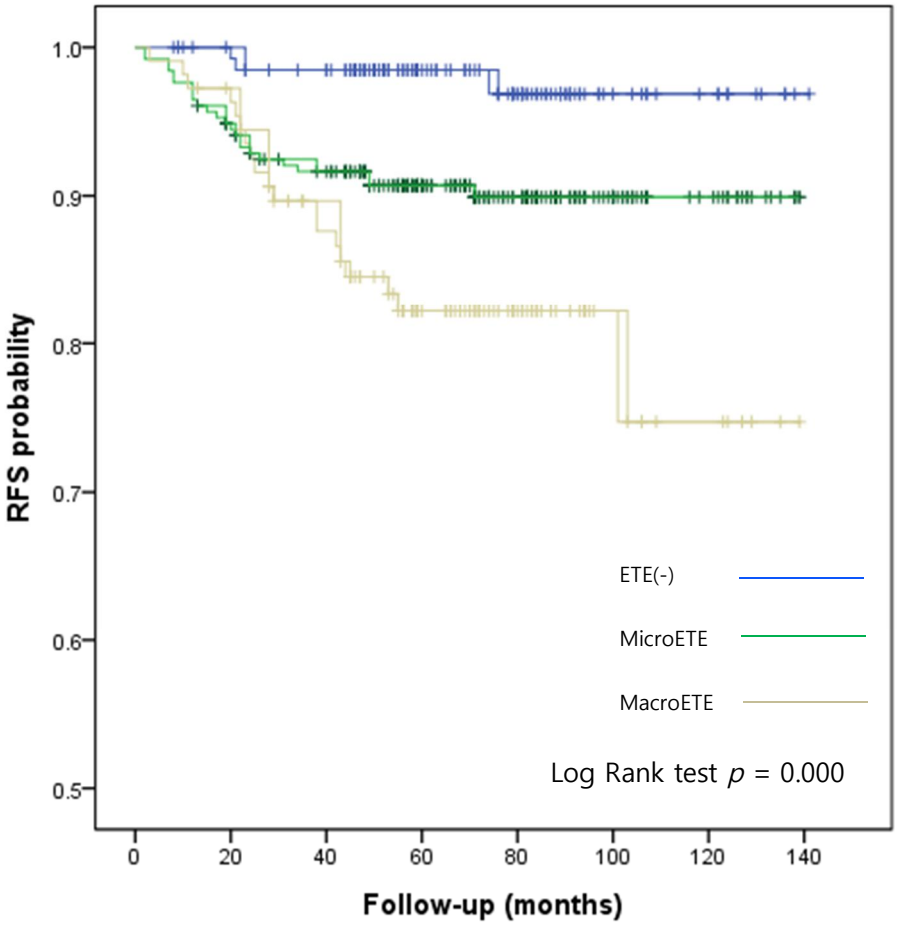

1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
Age at operation 60≥
60<
Log Rank test p = 0.005

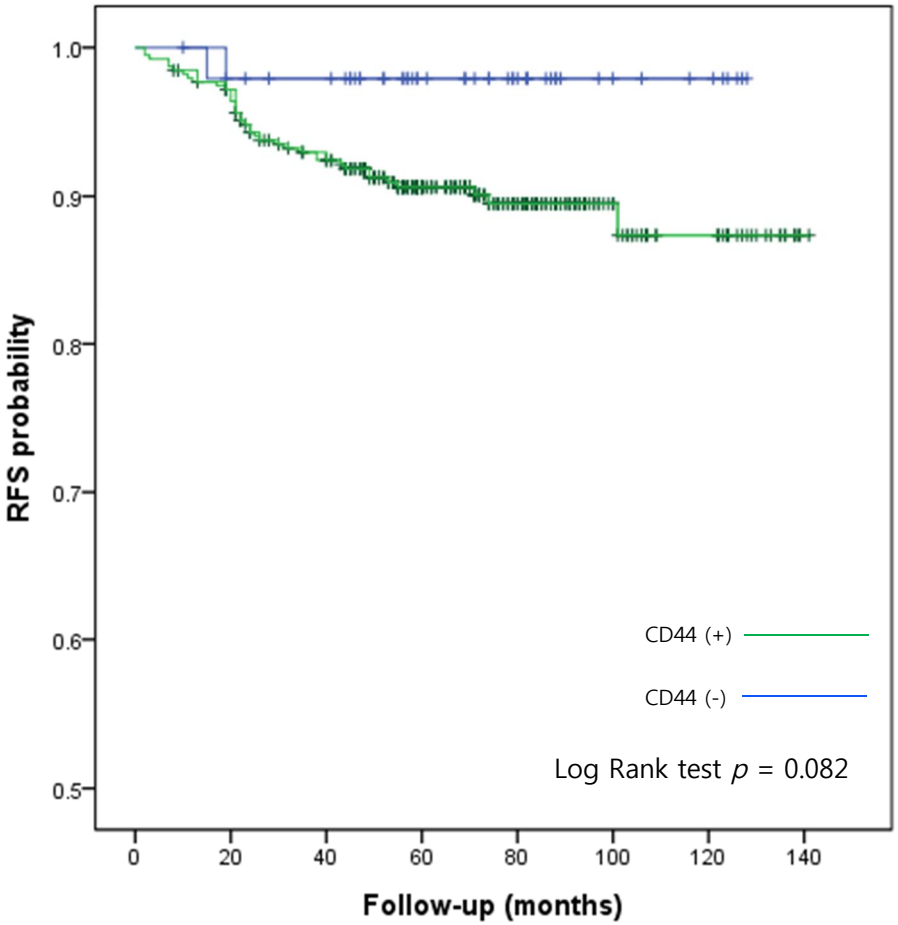

RFS probability
1.0
0.9
0.8
0.7
0.6
0.5
0
20
40
60
80
100
120
140
Follow-up (months)
cN1b(-)
cN1b(+)
Log Rank test p = 0.000

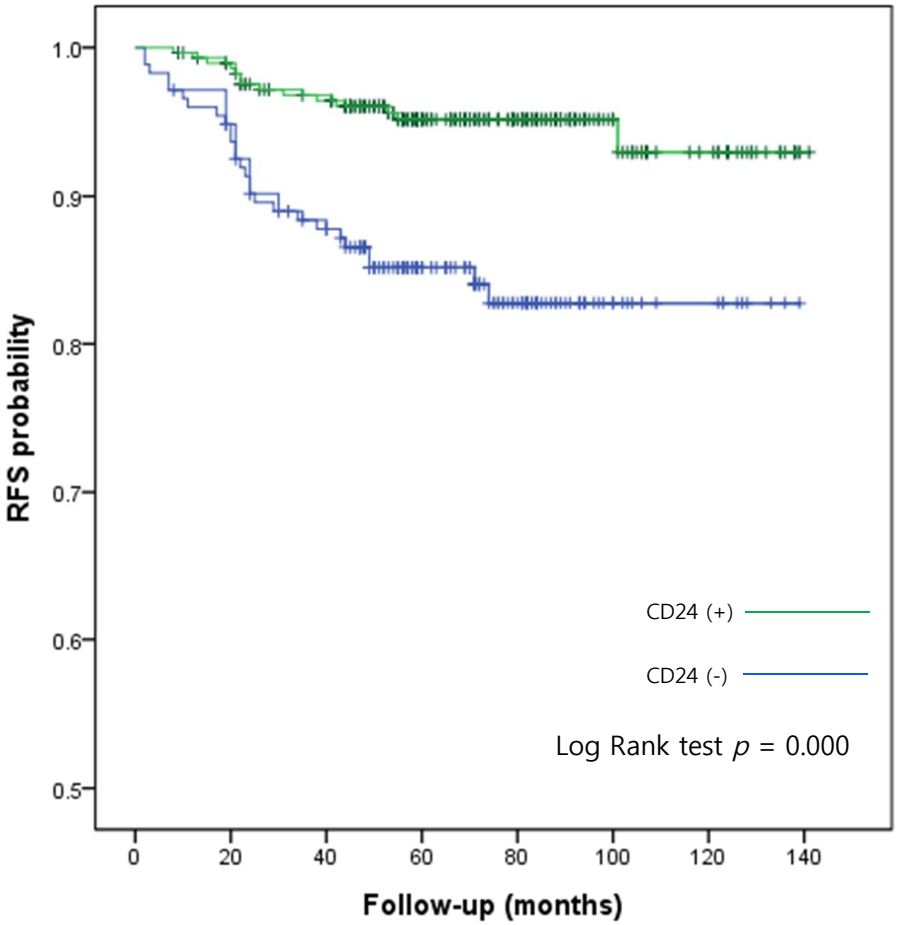

1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
pN1 ≤5
pN1 >5
Log Rank test p = 0.000

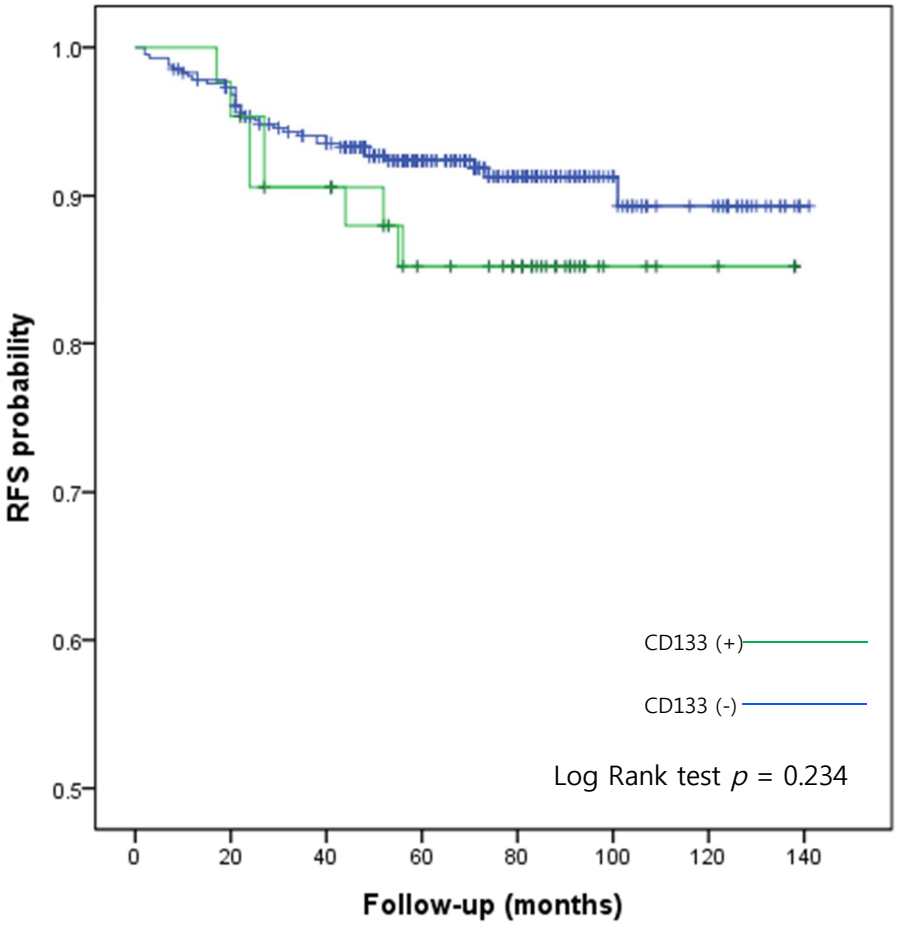

1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
pTumor size ≤2 cm
pTumor size >2 cm
Log Rank test p = 0.000

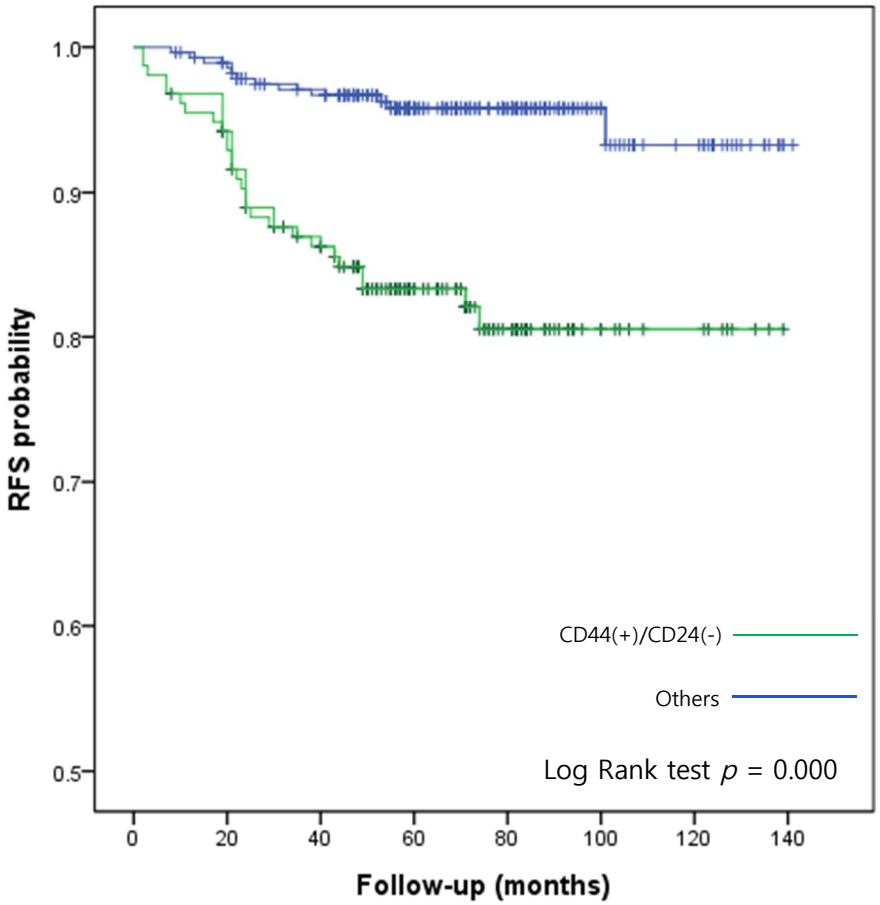

1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
Multifocality (+)
Multifocality (-)
Log Rank test p = 0.347

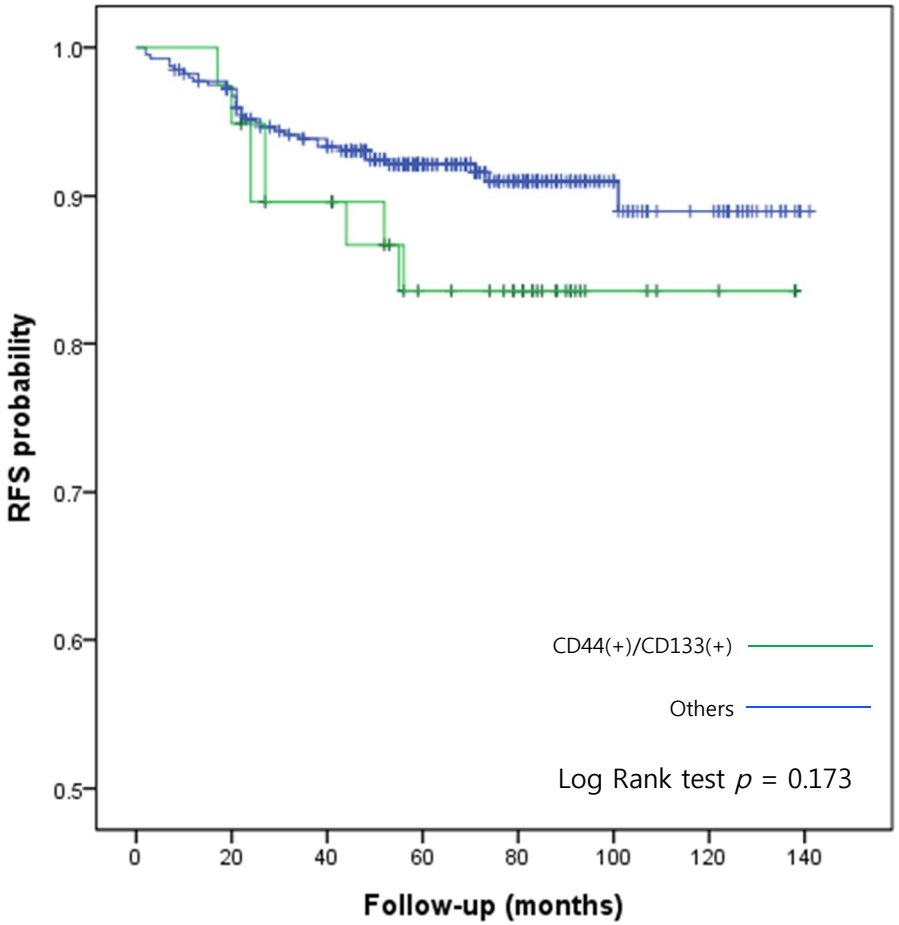

RFS probability
1.0
0.9
0.8
0.7
0.6
0.5
0
20
40
60
80
100
120
140
Follow-up (months)
ETE(-)
MicroETE
MacroETE
Log Rank test p = 0.000

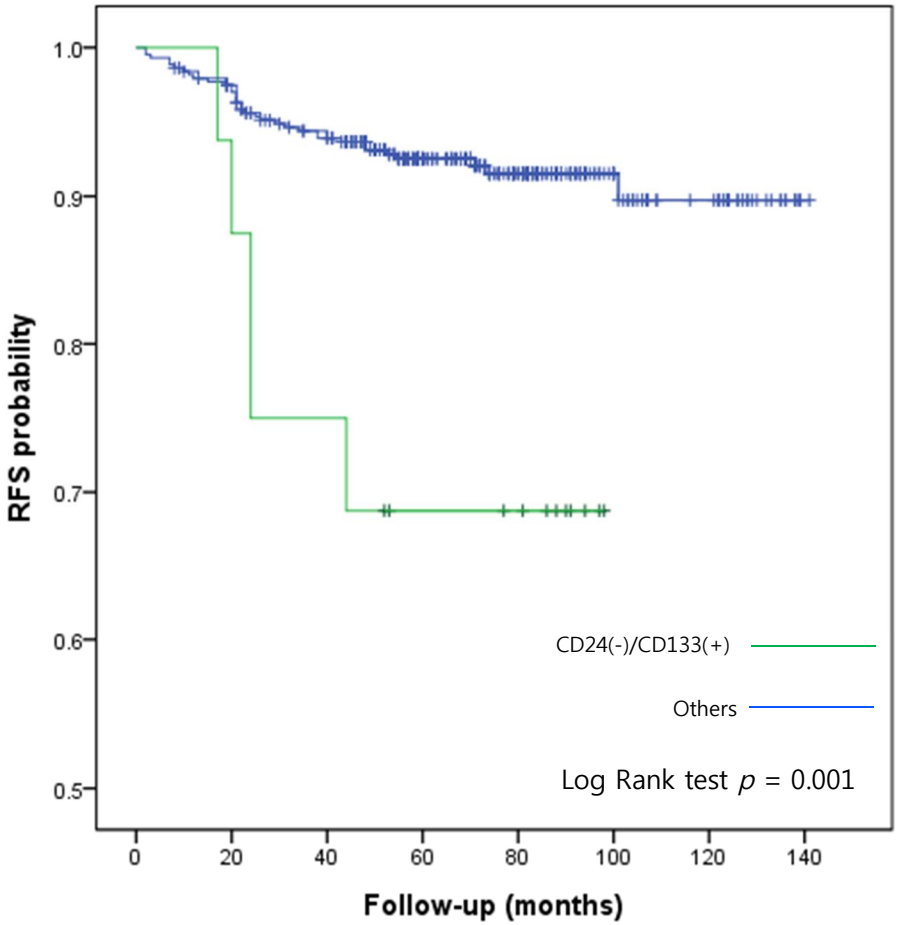

RFS probability
1.0
0.9
0.8
0.7
0.6
0.5
0
20
40
60
80
100
120
140
Follow-up (months)
CD44 (+)
CD44 (-)
Log Rank test p = 0.082

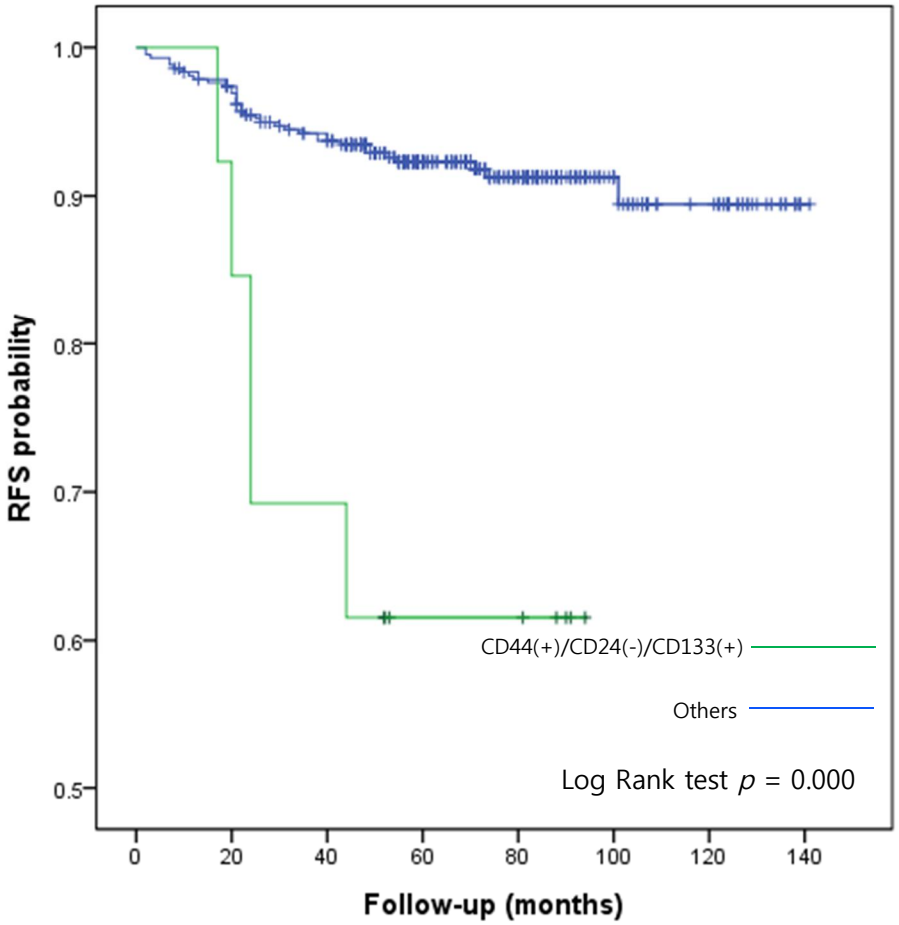

1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
CD24 (+)
CD24 (-)
Log Rank test p = 0.000


1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
CD133 (+)
CD133 (-)
Log Rank test p = 0.234


1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
CD44(+)/CD24(-)
Others
Log Rank test p = 0.000


1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
CD44(+)/CD133(+)
Others
Log Rank test p = 0.173


RFS probability
1.0
0.9
0.8
0.7
0.6
0.5
0
20
40
60
80
100
120
140
Follow-up (months)
CD24(-)/CD133(+)
Others
Log Rank test p = 0.001


1.0
0.9
0.8
0.7
0.6
0.5
RFS probability
0
20
40
60
80
100
120
140
Follow-up (months)
CD44(+)/CD24(-)/CD133(+)
Others
Log Rank test p = 0.000

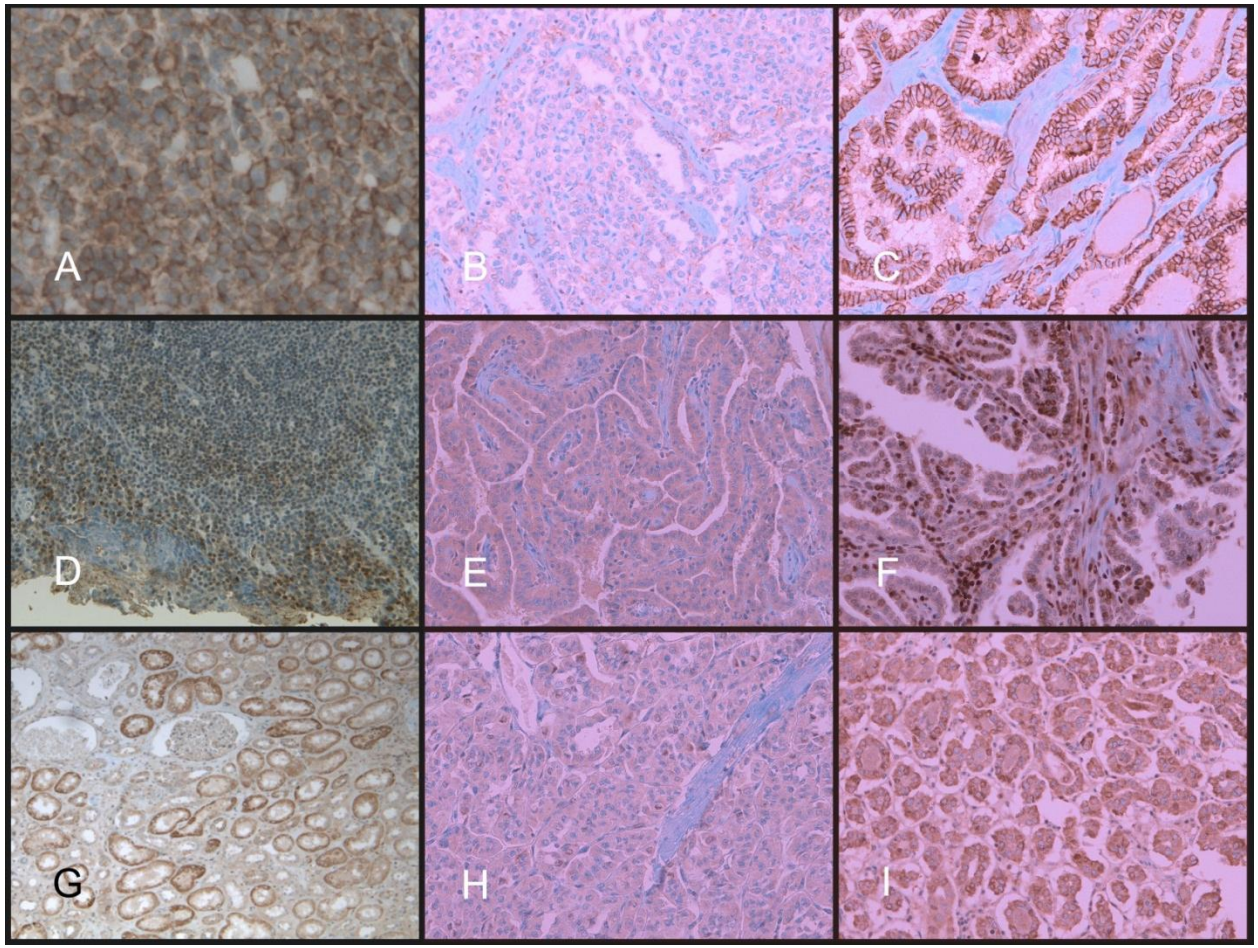


Figure 2 Immunohistochemical staining pattern of PTC

A: CD44 Positive Control - Human Tonsil B: CD44(-) C: CD44(+)

D: CD24 Positive Control - Human Tonsil E: CD24(-) F: CD24(+)

G: CD133 Positive Control – Human Kidney H: CD133(-) I: CD133(+)