Original article

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Calreticulin expression in neuroblastoma—a novel independent prognostic factor

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Background: Calreticulin (CRT), an endoplasmic reticulum protein, has been reported to be essential for the differentiation of neuroblastoma (NB) cells, suggesting that CRT may affect the tumor behavior of neuroblastoma. The aim of this study was to evaluate the association of clinicopathologic factors and patient survival with the expression of CRT in patients with NB.

Patients and methods: Sixty-eight NBs were investigated by immunohistochemical staining against CRT, and were divided into positive and negative immunostaining groups. Correlations between calreticulin expression, various clinicopathologic and biologic factors, and patient survival were studied. In seven tumor samples, CRT mRNAs and proteins were evaluated with real-time PCR and western blot, respectively, and correlated with immunohistochemical findings.

Results: Among 68 NBs, 32 (47.1%) showed positive CRT expression. Positive CRT immunostaining strongly correlated with differentiated histologies, as well as known favorable prognostic factors such as detected from mass screening, younger age (≤1 year) at diagnosis and early clinical stages, but inversely correlated with MYCN amplification. Kaplan–Meier analysis revealed that NB patients with CRT expression did have better survival. Multivariate analysis demonstrated CRT expression to be an independent prognostic factor. Moreover, CRT expression also predicted better survival in patients with advanced-stage NBs, and its absence predicted poorer survival in patients whose tumor had no MYCN amplification. The amount of CRT mRNAs and proteins in NB tumor samples tested correlated well with the immunohistochemical expressions.

Conclusions: CRT expression correlates with the differentiation of NB and predicts favorable survival, thereby suggesting CRT to be a useful indicator for planning treatment of NB.

Key words: calreticulin, immunohistochemistry, neuroblastoma, prognostic factor

Introduction

Neuroblastoma (NB), one of the most common pediatric cancers, is an embryonic cancer of the postganglionic sympathetic nervous system, which most commonly arises in the adrenal gland. The pathogenesis of NB remains obscure. NB cells have a great potential to differentiate into mature cells or to spontaneously regress. A large proportion of NBs identified from mass screening, as well as stage 4S tumors, later differentiate into mature histologies or spontaneously regress [1, 2]. In addition, many normally expressed molecular markers in embryonic neuroblastic cells, such as HNK-1, tyrosine hydroxylase, Trk-A and CD44, are found in NBs, suggesting that NB arises during the developmental stages of the embryonic sympathetic system [3, 4]. Furthermore, expression of apoptosis-related genes has been demonstrated in NB [5], and patients with NB tumors that showed more apoptosis had a better prognosis [6]. These studies suggest that failure of either differentiation or regression by apoptotic death of NB cells is critical for the development of clinical NB.

Calreticulin (CRT) is an endoplasmic reticulum protein with two major functions: molecular chaperoning and regulation of Ca^{2+} homeostasis [7]. Furthermore, CRT can also modulate cell adhesion, integrin-dependent Ca^{2+} signaling and steroid-sensitive gene expression outside the endoplasmic reticulum [7]. Although CRT has many physiologic functions in the cell, its role in pathologic conditions has been studied infrequently. Evidence suggests that CRT is linked to the biology of NB. CRT has been found on the surface of NB

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cells and is essential for neurite formation when the cells are induced to differentiate [8, 9]. In a NB cell line study, Johnson et al. showed that CRT protein levels increased markedly when the cells were induced to differentiate with dibutyryl c-AMP [10]. CRT has also been found to affect cell susceptibility to apoptosis and to be overexpressed in highly apoptotic regions of the embryo [11]. In addition, CRT has been shown to be essential for neural development in mice [12]. These lines of evidence give rise to the intriguing possibility that CRT may affect the differentiation and apoptosis of NB, and thus may have a role in the tumor behavior of this cancer.

In this study, CRT expression in NB tumors was studied by immunohistochemistry and related to clinicopathologic and biologic parameters to evaluate the importance of CRT in NB, and to analyze the prognostic relevance of CRT expression in this tumor. In addition, the mRNA and protein levels of CRT in tumor tissues were also quantified by real-time PCR and western blot, respectively, to compare with the immunohistochemical findings.

Materials and methods

Patients and treatment

Sixty-eight NB patients treated at the National Taiwan University Hospital from January 1991 to December 2002 were included in this study. Eight of these NB patients were identified by mass screening of urinary vanillylmandelic acid for infants. The eligibility criteria for patients to be enrolled in the study were the availability of sufficient tumor tissues for thorough studies and having complete follow-up. There were 36 males and 32 females. The median age at diagnosis was 2.5 years (range 0–11.5). Adrenal gland (37 cases) was the most common primary tumor site, followed by retroperitoneum (18 cases), mediastinum (six cases), neck (four cases) and pelvis (three cases). The histologic features of NB were classified into undifferentiated NB (UNB, 35 cases), differentiating NB (DNB, including poorly differentiated subtype, 20 cases), and ganglioneuroblastoma (GNB, 13 cases) according to the percentage and degree of differentiation of the NB cells using the criteria of the International NB Pathology Classification [13, 14]. Six patients had stage 1 tumors, 13 had stage 2 tumors, 10 had stage 3 tumors, 34 had stage 4 tumors and 5 had stage 4S tumors according to the International NB Staging System (INSS) [15]. Fifteen of the 68 NBs were demonstrated to have MYCN amplification by fluorescence *in situ* hybridization analysis of formalin-fixed paraffin-embedded tissues or fresh tumor single cells [16, 17]. All stage 1 tumors were treated by surgery alone, whereas the other tumors were treated with a combination of surgery and chemotherapy with or without autologous bone marrow transplantation according to the patient's risk grouping [2]. The median follow-up after diagnosis was 38 months (range, 1–144). The overall 5-year survival was 52.6%.

Immunohistochemical staining

CRT expression was assayed using an avidin–biotin complex immunoperoxidase staining technique on archival paraffin-embedded tissue specimens obtained before chemotherapy. The antibody to CRT gene product was affinity-purified, rabbit polyclonal immunoglobulin G—generated by synthetic peptide corresponding to the carboxy-terminal 6 amino acids (412–417) of CRT (Upstate, New York, NY, USA). Tissue sections (5 μm) of tumors were deparaffinized and rehydrated in a routine manner. After microwave pretreatment, the CRT antibody was then applied at a dilution of 1:150 overnight at 4°C. The N-Histofine Simple Stain MAXPO (Nichirei, Tokyo, Japan) was then applied for 30 min at room temperature. Diaminobenzidine was used for visualization and nuclei were counterstained with hematoxylin. One ganglioneuroma tumor with consistent CRT expression by immunohistochemistry was used as a positive control in each staining. Non-immunized rabbit serum was used as a negative control. Tumors with various differentiating histologies were included in each staining. The immunoreactivity of CRT was assessed by a single pathologist who was blinded to the clinical background of the patients. The immunoreactivity of CRT was recorded as follows: 'negative' indicated staining was absent throughout the specimen, and 'positive' indicated that brownish granular staining was present in cytoplasm of the NB or ganglion cells. To verify the specificity of the CRT antibody, a blocking peptide corresponding to the carboxy-terminal 17 amino acids (401–417) (5× of antibody; Santa Cruz, CA) was added along with the CRT antibody when carrying out the positive control staining to see if the immunostaining could be blocked specifically.

Real-time PCR

The mRNAs of CRT in NB tumor tissues were examined by real-time PCR as described previously [18] for comparison with the immunohistochemical expression of CRT. Total RNA from seven tumor samples (three UNBs, two DNBs and two GNBs) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After quantifying by O.D., 1 μg of total RNA was reverse-transcribed to cDNA using reverse transcriptase enzyme (NEB, Beverly, MA, USA). Real-time PCR was carried out using the iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA, USA) with SYBR-Green I (stock solution 10 000×, diluted at 1:25 000) as fluorescent dye enabling real time detection of PCR products according to the manufacturer's protocol. Gene-specific primers were used, and the specificity was tested under normal PCR conditions. Oligonucleotide primers for PCR were designed using Beacon Designer2 software (PREMIER Biosoft International, Palo Alto, CA, USA). The cDNA was subjected to real-time PCR using the primer pairs as follows: forward AAGTTCTACGGTGACGAGGAG, reverse GTCGATGTTCTGCTCATGTTTC for CRT; and forward GTGGTCTCCTCTGACTTCAAC, reverse TCTCTTCCTCTTGTGCTCTTG for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Cycling conditions were 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 60 s. For quantitation, the mRNA of *CRT* gene was normalized to the mRNA of the internal control gene *GAPDH*. For each tumor sample, data from three separate experiments were averaged.

Western blot analysis

CRT proteins in NB tumors were also examined by immunoblot analysis to compare with the results obtained by immunohistochemical study. The details of extraction of tumor tissue, electrophoresis and immunoblotting have been described previously [18]. In brief, the tumor tissues were homogenized. Extracts (50 μg of proteins) were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was pretreated with 5% skimmed milk and then incubated with the anti-CRT antibody (1:200). The bound antibody was labeled with horseradish peroxidase-conjugated anti-rabbit antibody, and visualized by immersion in a 4-chloro-1-naphthol reagent (4CN Plus, Perkin-Elmer, Boston, MA, USA) as substrate. To test the specificity of anti-CRT antibody used in immunohistochemical study, competition assay was carried out in which the specific blocking peptide (5× of antibody) was added along with the CRT antibody during immunoblotting. Cell lysates from untreated

and all-trans retinoic acid-treated NB cell line (Neuro-2A, American Type Culture Collection CCl-131) [19] were used as positive control for immunoblot analysis of CRT.

Statistics

The statistical analyses were carried out with SPSS 10.0 for Windows software. Associations between pairs of categorical variables were assessed with Pearson's chi-square test. Survival probabilities in various subgroups were estimated using the Kaplan–Meier method, and analyzed by log-rank tests. The influence of each variable on survival was assessed by the multivariate Cox proportional hazard model. All statistical tests were two-sided and a P value of 0.05 or less was considered to be statistically significant.

Results

CRT expression profiles for NB tissues

In the positive control staining of a ganglioneuroma, positive CRT staining was seen in ganglion cells, and localized in the cytoplasm consistent with endoplasmic reticulum location of CRT (Figure 1A). When the specific blocking peptide was added along with the anti-CRT antibody, the immunostaining was blocked completely (Figure 1B). Among 68 NB tumors, 32 (47.1%) showed positive expression of CRT in NB or ganglion cells. The CRT-positive staining was mainly seen in DNB cells or GNB cells (Figure 1C–E). Eighteen of

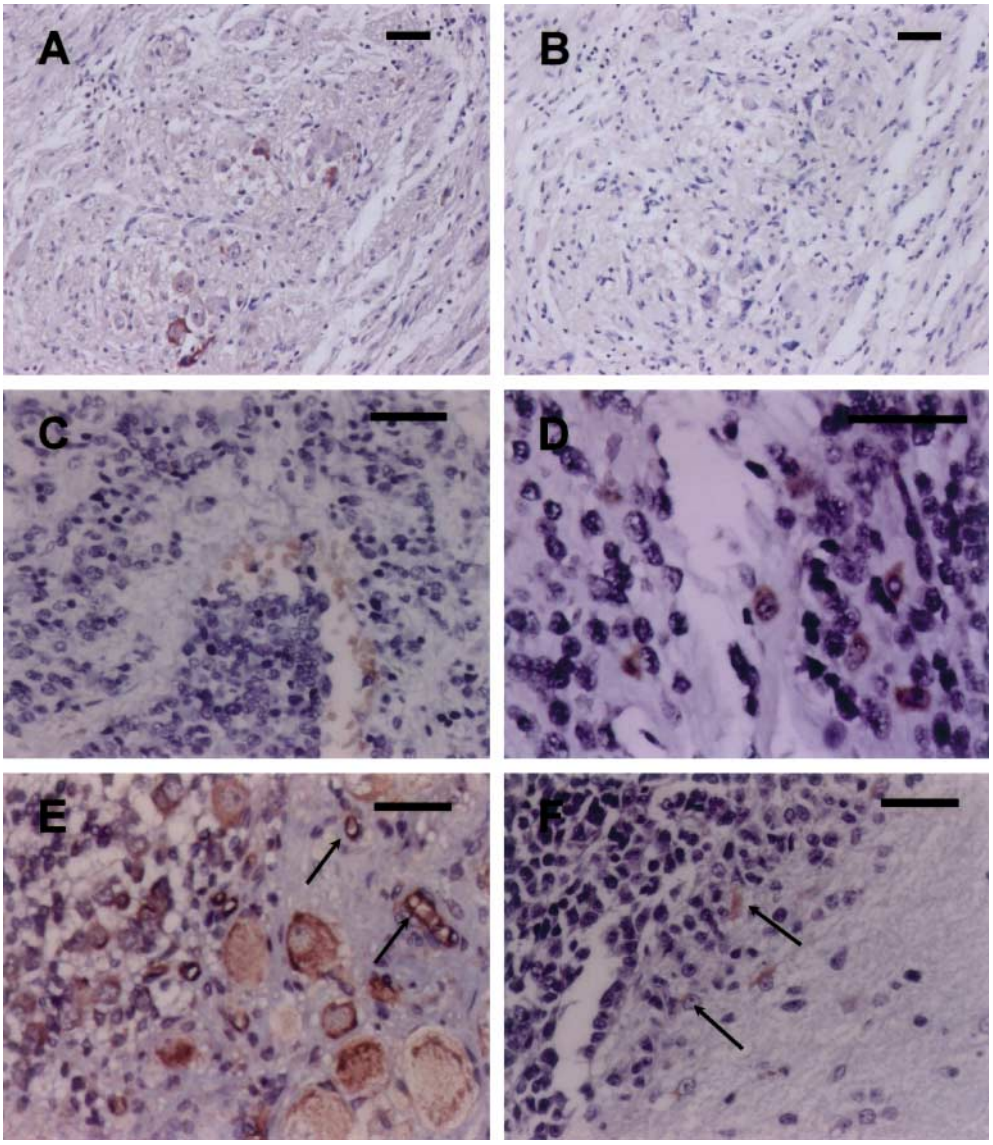

Figure 1. Immunohistochemical study of calreticulin expression. (**A**) Positive control staining of a ganglioneuroma. (**B**) Positive control staining blocked by specific peptide. (**C**) Undifferentiated neuroblastoma (NB) shows negative staining. (**D**) Differentiating NB shows positive staining in differentiating NB cells. (**E**) Ganglioneuroblastoma shows positive staining in differentiating NB cells, ganglion cells and endothelial cells (arrows). (**F**) Undifferentiated NB with brain metastasis shows positive staining in brain neurons (arrows) but negative staining in NB cells (left upper part). Scale bars = 100 μm.

the 32 NB tumors (56.3%) also had positive CRT-expression in the endothelial cells. However, no tumor had positive staining of Schwann cells. Three tumors having CRT expression only in endothelial cells but not in neuroblastic cells were designated as having negative CRT expression in the analysis. One UNB with brain metastasis showed negative CRT staining in the tumor, whereas the adjacent normal neurons did express CRT (Figure 1F). Furthermore, normal sympathetic ganglions and the adrenal medulla also showed positive CRT staining in immunohistochemical studies (data not shown).

CRT mRNA and protein levels in seven NB tumors were evaluated by real-time PCR and western blot to compare with the results of immunostaining (Figure 2A, lanes a–g). The results of real-time PCR were quite compatible with those of immunostaining. Cases with differentiated histologies (DNB or GNB) showed high expression of CRT mRNA and positive immunostaining (Figure 2A, lanes a–c, f). On the other hand, cases with UNB had low expression of CRT mRNA and negative immunostaining (Figure 2A, lanes d, e, g). In western blot study using the same antibody as used in immunohistochemistry, a single band of approximately 55 kDa corresponding to the reported size of CRT could be detected in positive control Neuro-2A cells as well as in cases with high mRNA expression and positive immunostaining (Figure 2A, D0, D5 and lanes a–c). However, there were a few cases showing a discrepancy between immunoblot and immunostaining results (Figure 2A, lanes e–g). Since the stromal cells of differentiated histologies had either no or very low expression of CRT, they could result in a dilution effect of proteins in the tumor lysates (Figure 2A, lane f). On the other hand, since

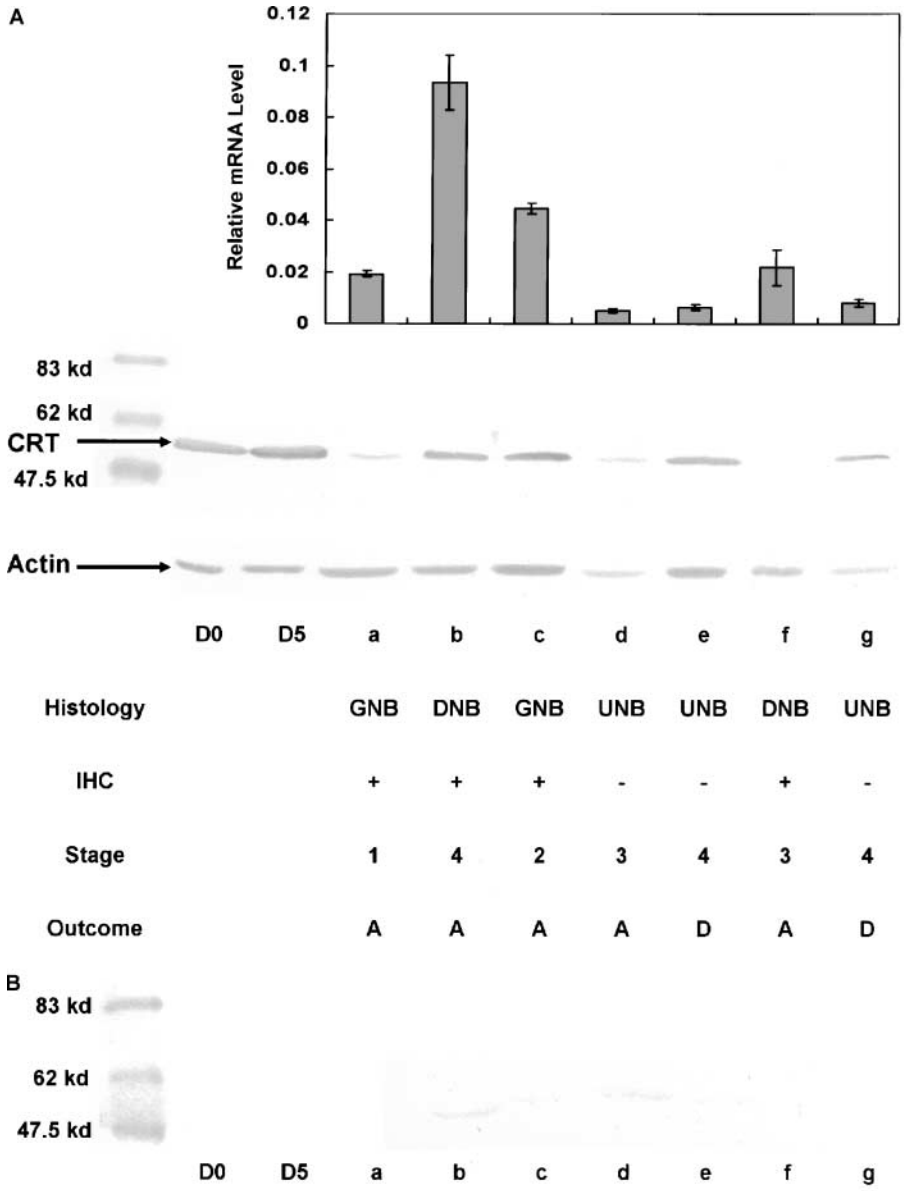

Figure 2. (**A**) Real-time PCR and western blot analysis of calreticulin (CRT) mRNA and protein in neuroblastoma cell lines and tumors. (**B**) Competition assay. D0, untreated Neuro-2A cells; D5, Neuro-2A cells treated with all-trans retinoic acid for 5 days; GNB, ganglioneuroma; DNB, differentiating neuroblastoma; UNB, undifferentiated neuroblastoma; A, alive; D, dead; IHC, immunohistochemistry.

cases with undifferentiated histology usually had a quite compact NB cell population, despite the fact that individual NB cells had a low CRT protein level, the total protein levels might be high in the whole tumor lysate (Figure 2A, lanes e, g). In the cell line studies, differentiated Neuro-2A cells induced by retinoic acid treatment for 5 days (Figure 2A, D5) had higher CRT protein levels than untreated cells (Figure 2A, D0). However, untreated cell lysates still had a significant level of CRT protein. These results indicated that in studying such a heterogenous tumor as NB, immunohistochemistry had the advantage of identifying the specific cells of interest and evaluating the overall tumor quality simultaneously when compared with PCR and western blot. The specificity of anti-CRT antibody used in immunohistochemistry was again confirmed by a competition assay, in which the CRT immunoblotting was blocked completely by the specific antigenic peptide (Figure 2B).

CRT expression and clinicopathological and biological features

The relationship between CRT expression and clinicopathological and biological variables of NB is summarized in Table 1. The percentage of positive CRT immunostaining increased as the tumor histology became differentiated with 12 of 20 (60%) differentiating NBs and 12 of 13 (92.3%) GNBs showing positive staining, whereas only eight of 35 (22.9%) undifferentiated NBs had positive staining ($P<0.001$). Positive CRT immunostaining was also frequently seen in tumors detected from mass screening ($P=0.022$), as well as in tumors of infants ($P=0.016$) and early clinical stages (stage 1, 2, 4S) ($P<0.001$). However, there was an inverse correlation between CRT expression and MYCN amplification ($P=0.003$), with only two tumors showing both MYCN amplification and positive CRT expression.

Survival analysis

Kaplan–Meier analysis revealed that 5-year survival rates in patients with positive or negative CRT expression were 81.9% and 28.4%, respectively (Figure 3). Positive CRT expression predicted a significantly better survival ($P<0.001$, log-rank test). Univariate analysis further showed that younger age (≤1 year) at diagnosis, early clinical stages and differentiated tumor histology correlated strongly with better survival, whereas MYCN amplification predicted a very poor outcome (Table 2). In multivariate analysis, CRT expression, in addition to clinical stage and MYCN amplification, was demonstrated to be an independent prognostic factor of NB (Table 2).

For further understanding of the significance of CRT expression in the prognostic discrimination, the survival of NB patients was stratified according to the CRT expression, clinical stage and MYCN amplification (Figure 4). In 24 NBs of early stages (stage 1, 2 or 4S), 20 had positive CRT expression and four had negative CRT expression. All of them survived except for two surgical mortalities. In 44 patients

Table 1. CRT expression and clinicopathological and biological factors

	Cases	Positive CRT expression (%)	*P* value
Mass screening			
Yes	8	7 (87.5)	0.022[a]
No	60	25 (41.7)	
Sex			
Male	36	16 (44.4)	0.647
Female	32	16 (50.0)	
Age at diagnosis			
≤1 year	22	15 (68.2)	0.016
>1 year	46	17 (37.0)	
INSS clinical stage			
1	6	5 (83.3)	<0.001[b]
2	13	11 (84.6)	
3	10	3 (30.0)	
4	34	9 (26.5)	
4S	5	4 (80.0)	
Primary tumor site			
Adrenal	37	14 (37.8)	0.096
Extra-adrenal	31	18 (58.1)	
Tumor histology			
Undifferentiated NB	35	8 (22.9)	<0.001
Differentiating NB	20	12 (60.0)	
GNB	13	12 (92.3)	
MYCN			
Amplified	15	2 (13.3)	0.003[a]
Non-amplified	53	30 (56.6)	

[a]Fisher exact test.
[b]Stage 1, 2, 4S versus 3, 4.
CRT, calreticulin; NB, neuroblastoma; GNB, ganglioneuroblastoma; INSS, International NB Staging System.

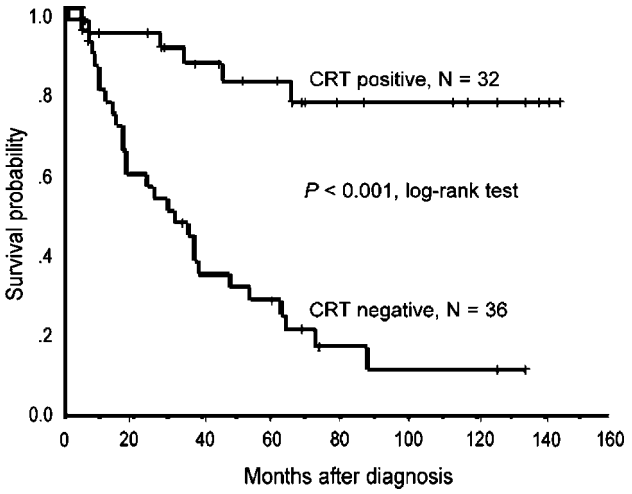

Figure 3. Overall survival according to calreticulin (CRT) expression determined by immunohistochemistry.

Table 2. Clinicopathological and biological factors affecting survival rate

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Age at diagnosis: ≤1 year versus >1 year	3.531	1.362–9.154	0.009	1.175	0.378–3.648	0.780
Clinical stage: early (1, 2, 4S) versus advanced (3, 4)	14.172	3.376–59.498	<0.001	7.741	1.354–44.264	0.021
MYCN: non-amplified versus amplified	5.864	2.720–12.642	<0.001	2.550	1.143–5.689	0.022
CRT expression: positive versus negative	7.120	2.925–17.329	<0.001	2.838	1.033–7.793	0.043
Histology: differentiated[a] versus undifferentiated	2.239	1.118–4.487	0.023	1.261	0.581–2.736	0.557
Primary tumor site: extra-adrenal versus adrenal	1.591	0.792–3.196	0.192	ND		

[a]Including differentiating neuroblastoma and ganglioneuroblastoma.

CRT, calreticulin; RR, relative risk; 95% CI, 95% confidence interval; ND, not done.

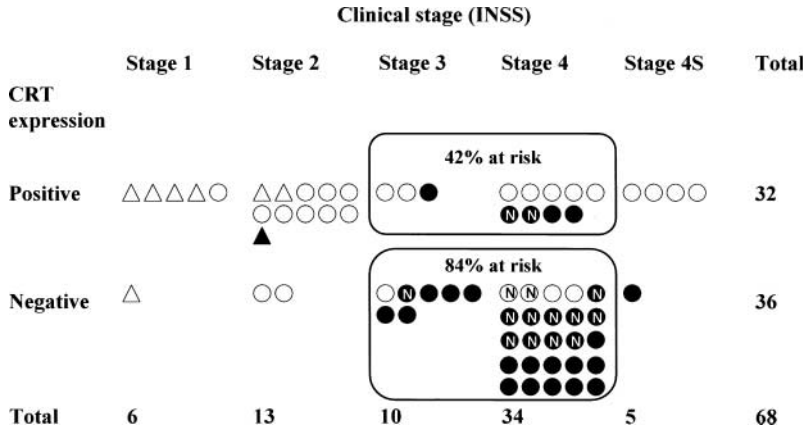

Figure 4. Prognostic categories of calreticulin (CRT) expression, clinical stage (according to International Neuroblastoma Staging System, INSS) and MYCN status in 68 neuroblastoma patients. △, tumors identified by mass screening; ○, tumors identified clinically. 'N' in a circle represents MYCN amplification. Open symbols represent survivors; filled symbols (▲ and ●) represent patients who died. Risk for unfavorable biology was calculated as the ratio of deceased cases in each subgroup circled.

with advanced-stage disease (stage 3 or 4), the prognosis could be clearly distinguished by CRT expression. In the category of advanced stage and positive CRT expression, only five of 12 patients (42%) died of their disease. In the category of advanced stage and negative CRT expression, 27 of 32 patients died, indicating an 84% risk of an unfavorable outcome. Therefore, positive CRT expression predicted a favorable chance of survival in patients with advanced-stage disease (Figure 5A, $P = 0.007$, log-rank test). Among 15 NBs with MYCN amplification, only two had positive CRT expression. All 15 patients with MYCN amplification died of NB except two who were alive with residual tumor at 5 and 7 months follow-up. On the other hand, among 53 NBs without MYCN amplification, positive CRT expression distinguished the patients with favorable outcomes from those with unfavorable outcomes (Figure 5B, $P < 0.001$, log-rank test). In the category of normal MYCN copy number and positive CRT expression, only four of 30 patients (13%) died, whereas in the category of normal MYCN copy number and negative CRT expression, 17 of 23 patients (74%) died of their disease.

Discussion

Recent advances in understanding the biology and genetics of NB have allowed a risk-group-based therapy [2, 20], suggesting that detailed biologic studies of tumor behavior are critical to the treatment of NB [20]. This study was designed to determine whether immunohistochemical expression of CRT in NB was associated with clinicopathologic and biologic parameters and whether the expression was useful for predicting outcome. Our study demonstrated clearly that CRT expression was an independent prognostic factor for the survival of NB patients. Clinical stage and MYCN amplification are two well-known prognostic factors of NB [2, 20]. Nevertheless only 35% (24/68) of NB patients were detected at early stages and fewer patients (22%, 15/68) were found to have tumors with MYCN amplification. In addition, since not all patients with advanced-stage disease had very poor prognoses (5-year survival rate 32%, Table 2) and not all patients whose tumors had no MYCN amplification had very good prognoses (5-year survival rate 67%, Table 2), these two categories of patients were actually large groups of patients with

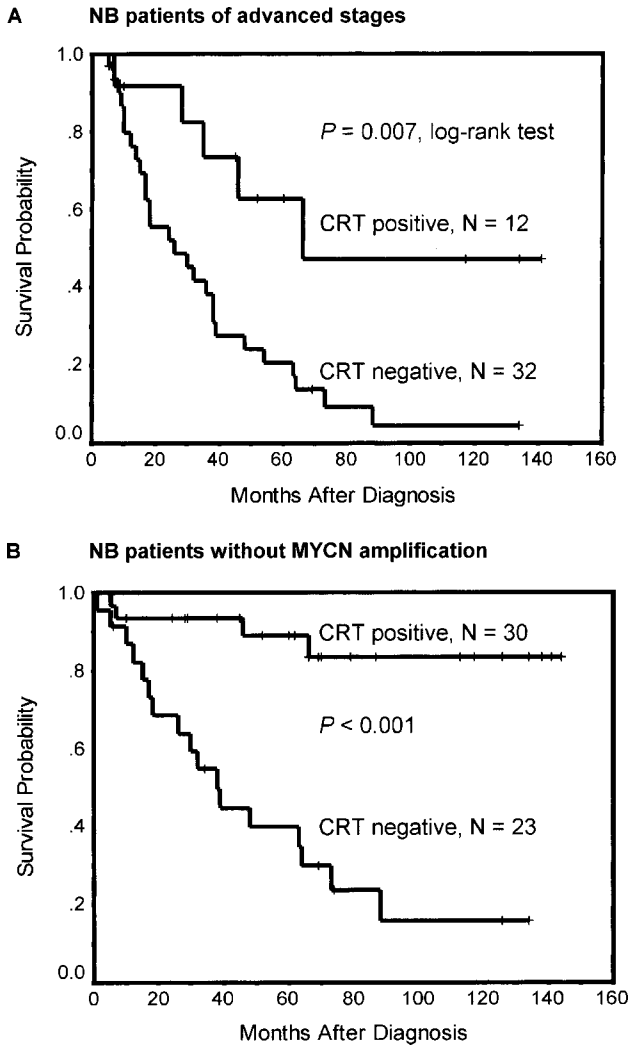

Figure 5. Kaplan–Meier survival analysis according to calreticulin (CRT) expression (determined by immunohistochemistry) in patients with neuroblastoma (NB). Positive CRT expression predicts favorable prognoses in (**A**) patients with advanced diseases (stage 3 and 4) and (**B**) patients with tumors without MYCN amplification.

clinical heterogeneity. Additional factors were required to further distinguish the prognoses of these patients. Our results showed that among patients with advanced-stage disease and those with tumors without MYCN amplification, positive CRT expression distinguished the patients with favorable prognoses from those with unfavorable prognoses (Figure 5). These results indicate that immunohistochemical study of CRT may provide complementary prognostic information, which in turn may be helpful in the determination of the most appropriate intensity of therapy.

Our immunohistochemistry studies showed that the percentage of positive CRT expression was high in NBs of infants and patients in earlier stages of disease, as well as in mass-screened NBs. NB tumors with clinical characteristics of younger age (≤1 year), early clinical stages, and detected by mass screening have a strong tendency to differentiate or regress spontaneously [1, 21]. This evidence indicates that NB tumors with positive CRT expression may be more likely to differentiate or regress spontaneously. In fact, there was a strong correlation between CRT expression and histologic grade of differentiation, yet only CRT expression but not histologic grade of differentiation was demonstrated to be an independent prognostic factor. This result supports the notion that CRT negatively regulates the growth of NB cells by affecting more than cell differentiation alone. In addition, positive CRT immunostaining was seen in normal brain neurons, adrenal medulla and sympathetic ganglia. This finding suggests not only that CRT expression is involved in the differentiation and regression of NB, but also that up-regulation of CRT is required for the normal development of neuronal cells.

Expression of Trk-A, a nerve growth factor (NGF) receptor, has been shown to be associated with the differentiation and death of the NB cells, as well as favorable prognosis of NB patients [22]. Trk-A, after activation by NGF, may promote intracellular signaling cascades, including the Ras/ERK protein kinase pathway, the PI3K/Akt kinase pathway and PLC-γ1 [23]. Activated PLC-γ1 acts to hydrolyze phosphatidylinositides to generate diacylglycerol, which may further activate the PKC-δ [23]. PKC-δ in turn is required for activation of the ERK cascade and for neurite outgrowth [23]. Interestingly, it has been shown that CRT is a substrate and binding protein for all PKC isoforms, suggesting that CRT plays an important role in the common PKC activated signaling pathway [24]. Thus, it is conceivable that CRT may participate in the process of Trk-A-mediated NB cell differentiation and death. It would be interesting to study the association between Trk-A and CRT expression in NB.

It was very interesting to find a specific expression of CRT in the endothelial cells by our immunohistochemistry study. A parallel expression of CRT in both endothelial cells and neuroblastic cells was also observed. More than half of the tumor samples that had positive CRT staining in their neuroblastic cells also had positive staining in their endothelial cells. Three tumors had CRT expression only in endothelial cells but not in neuroblastic cells and were designated as having negative CRT expression in the analysis. Two of these three tumors were GNB, and the remaining one was an UNB detected by mass screening. These findings indicate that CRT expression in the endothelial cells may also be relevant to the differentiation and regression of NB. However, the relationship between the expression of CRT in endothelial cells and neuroblastic cells is not known. CRT has been shown to be an anti-angiogenic factor that may inhibit the growth of endothelial cells [25], and has been used as a target of gene therapy for cancer [26]. Interestingly, it has also been shown that inhibition of angiogenesis may induce differentiation and apoptosis in NB [27]. The association of CRT expression in endothelial cells and neuroblastic cells in our studies may suggest that CRT is a potential target for the treatment of NB by both inhibiting endothelial cells and promoting differentiation and regression of NB cells.

CRT is a unique endoplasmic reticulum protein that affects many cellular functions; therefore, it is possible that it is

involved in many pathologic conditions, especially in cancers. CRT has been shown to exist in the nuclear matrix of human hepatocellular carcinoma and various carcinoma cell lines [28], and overexpressed in human breast carcinoma [29]. However, the role and importance of CRT in these cancers are not known. Our study demonstrates for the first time that expression of CRT in NB correlates with a differentiated histology and better outcome. Detection of CRT expression in tumor tissues may be of potential use as a predictive marker for NB. However, due to the limited sample size from one single institute of this cohort, additional studies with larger patient populations are required to further elucidate the prognostic significance of CRT expression in NB.

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