行政院國家科學委員會專題研究計畫　成果報告

Calreticulin 在神經母細胞瘤分化及凋亡時所可能參與之機制

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-273-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院外科

計畫主持人：許文明
共同主持人：陳維昭，郭明良

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 10 月 5 日

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※

※Calreticulin 在神經母細胞瘤分化及凋亡時所可能參※
※與之機制 ※
※The Role of Calreticulin in the Differentiation and ※
※Apoptosis of Neuroblastoma ※

※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC　92－2314－B－002－273－
執行期間：92 年　8 月　1 日至　93 年　7 月 31 日

計畫主持人：許 文 明
共同主持人：陳 維 昭
郭 明 良

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：台灣大學附設醫院外科部

中　華　民　國　93　年　8　月　25　日

行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 92－2314－B－002－273－
執行期限：92 年 8 月 1 日至 93 年 7 月 31 日

主持人：許 文 明　台灣大學附設醫院外科部
共同主持人：陳 維 昭　台灣大學附設醫院外科部
郭 明 良　台灣大學醫學院毒理學研究所

中文摘要

Calreticulin(CRT)在神經母細胞瘤分化及凋亡扮演相當重要的角色。然而 CRT 是透過何種機制參與神經母細胞瘤的分化及凋亡則仍需加以釐清。本計畫進一步探討 CRT 表現與 calnexin、Trk-A、GRP78 及 N-*myc* 的相關性。結果顯示 68 個神經母細胞瘤均表現 calnexin，有 32 個表現 CRT、35 個表現 Trk-A、有 40 個表現 GRP78，15 個腫瘤有 N-*myc* 放大。CRT 的表現和 Trk-A、GRP78 呈強烈正相關(P<0.001)，但與 N-*myc* 放大呈負相關(P=0.003)。CRT 和 Trk-A、GRP78 的表現對於預後的預測有加成效果，CRT 的表現對於沒有 N-*myc* 放大的病人亦可做為預後指標。CRT 可能參與 Trk-A 及 N-*myc* 在神經母細胞瘤的致病機轉，且和 GRP78 有協同作用。

關鍵詞：Calreticulin、神經母細胞瘤、細胞分化、細胞凋亡、Calnexin, Trk-A、GRP78、N-*myc*

Abstract:

Previous studies showed that calreticulin (CRT) played an important role in the differentiation and apoptosis of neuroblastoma (NB). However, the mechanism it might be involved in these processes are not clear. This study showed that in 68 NBs, all expressed calnexin, 32 expressed CRT, 35 expressed Trk-A, and 40 expressed GRP78. The expression of CRT strongly correlated with Trk-A and GRP78, but inversely correlated with N-*myc* amplification. There is a synergistic effect between CRT and Trk-A and GRP78 on the patient survival. CRT might be involved in the signaling pathway of Trk-A and N-*myc* of NB. CRT might also share a common role with GRP78 in NB.

Keywords: Calreticulin, Neuroblastoma, Cell differentiation, Apoptosis, Calnexin, Trk-A, GRP78, N-*myc*

Background and Purpose:

Calreticulin (CRT) is an endoplasmic reticulum protein with two major functions: molecular chaperoning and regulation of Ca^{2+} homeostasis.[1] CRT can also modulate cell adhesion, integrin-dependent Ca^{2+} signaling[1] and steroid sensitive gene expression.[2,3] Evidence suggests that CRT is linked to the biology of neuroblastoma (NB). CRT has been found on the surface of NB cells and it is essential for neurite formation when the cells are induced to differentiate.[4,5] CRT has also been found to affect cell sensitivity to apoptosis and to be over-expressed in highly apoptotic regions of the embryo.[6] In addition, CRT has been shown to be essential for neural development in mice.[7] These lines of evidence give rise to the intriguing possibility that CRT may contribute to the differentiation and apoptosis of NB, and thus may have a role in the tumor behavior of this cancer. Our previous immunohistochemistry study showed that CRT expression in NB strongly correlated with the histologic grade of differentiation and was an independent favorable prognostic factor of NB. However, the exact role of CRT in NB remained unknown.

The aim of this study is to explore the possible mechanisms that CRT may be involved in the differentiation and apoptosis of NB cells. CRT is basically a chaperon protein and a stress protein.[1] Two proteins, calnexin (a chaperone protein) and GRP78 (a stress protein), are chosen to evaluate possible associated changes with CRT. Calnexin is the major chaperone protein in the ER,[8] if CRT plays a role in NB cells via its chaperone function, there might be associated changes of calnexin with CRT. Likewise, if CRT plays a role via its function as a stress protein, then GRP78, another major stress protein,[9] might have associated changes. In addition, since the expression of CRT is associated with the differentiaton and regression of NB, it may have some relationship with another well-known prognostic proteins of NB, Trk-A (a nerve growth factor receptor).[10]

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 vanillyl-mandelic acid for infants. There were 36 males and 32 females. The median age at diagnosis was 2.5 years (range, 0 - 11.5 years). Adrenal gland (37 cases) was the most common primary tumor site, followed by retroperitoneum (18 cases), mediastinum (6 cases), neck (4 cases) and pelvis (3 cases). The histologic features of NB were classified into

undifferentiated NB (including poorly differentiated subtype, 34 cases), differentiating NB (20 cases), and ganglioneuroblastoma (GNB, 14 cases) according to the percentage and degree of differentiation of the NB cells.[11] 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).[12] 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.[13] The median follow-up after diagnosis was 38 months (range, 1-144 months). The overall 5-year survival was 52.6%.

Immunohistochemistry:

Formalin-fixed and paraffin-embedded tissue sections (5 μm) of tumors are deparaffinized and rehydrated, and endogenous peroxidase activity is blocked by incubation in 3% H_2O_2 for 15 minutes. Slides are microwaved for 15 minutes in 100 mM sodium citrate, pH 6.0. A rabbit anti-human CRT polyclonal antibody (Upstate, New York) is then applied at a dilution of 1:150 overnight at 4ºC. After rinsing with PBS, the N-Histofine Simple Stain MAXPO (Nichirei, Tokyo, Japan) is applied for 30 minutes at room temperature. Diaminobenzidine is used for visualization. One GN tumor with consistent CRT expression by immunohistochemistry is used as a positive control in each staining. The omission of the primary CRT antibody is used as a negative control. Tumors with various differentiating histologies are included in each staining. The immmunoreactivity of CRT and differentiating states of NB are assessed by 2 independent observers who are blinded to the clinical backgrounds of the patients. The immunoreactivity of CRT is recorded as follows: "negative" indicated no staining or weak staining of single neuroblastic cells (less than 5% of NB or ganglion cells), and "positive" indicated that at least 6% of the neuroblastic cells are stained. The immunohistochemical analysis of calnexin, GRP78, and Trk-A is performed with the similar procedures as described above except the primary antibodies are substituted with antibodies to calnexin, GRP78, and Trk-A(Upstate, New York).

Analysis of N-myc amplification by Fluorescence in situ hybridization (FISH)

The FISH procedure was performed using paraffin sections (4-6μm) as described elsewhere with modification.[14] Briefly, the tissue sections were deparaffinized with Hemo-DE (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and pretreated with 0.2 N HCL and protease. The nuclei on the slide was fixed with formalin and denatured in 70% formamide(Sigma, Missouri, USA)/2x standard saline citrate (SSC) (aMersco, Solon, Ohio, USA) at 75˚C for 30 minutes, and was then dehydrated through an ethanol series of 70%, 100% for 5 minutes. The hybridization buffer containing 10 ng aliquot of N-myc probe (spectrum orange, Vysis Inc., Downers Grove, Illinois, USA) was denatured for 5 minutes at 75˚C, chilled on ice, and then applied on the slide. Hybridization was performed overnight at 37˚C under sealed coverslip. The slide was then washed in 0.4x SSC/0.3% NP-40 (Vysis Inc.) at 73˚C for 2 minutes, followed by 2x SSC/0.1% NP-40 for 1 minute. The slide was counterstained with 10 μl DAPI (0.5g/ml 4'6-diamidino-2-phenyl indole dihydrochloride, Vysis Inc.) to reveal the morphology of nuclei by blue fluorescent signals. The signals representing the N-myc gene were counted for 100 cells on one slide. Cells with clusters or more than 6 grains of red fluorescence were designated as N-myc amplification. Tissues were analyzed in 3 separate experiments using different lots of N-myc probe. Leukocytes from a normal male donor were used as controls for FISH.

Results:

Among the 68 NB tumors, 68 (100%) tumors showed positive calnexin expression, 32 (47.1%) showed positive CRT expression, 35 (51.5%) showed positive Trk-A expression, 40 (58.8%) showed positive GRP78 expression, and 15 tumors showed N-*myc* amplification. CRT expression strongly correlated with the Trk-A and GRP78 expression (both P<0.001), but inversely correlated with N-*myc* amplification (P = 0.003). CRT, Trk-A, and GRP78 expression predicted a favorable outcome in NB patients (all P<0.001), whereas N-*myc* amplification predicted a very poor outcome (P<0.001). CRT and Trk-A or GRP78 expression had synergistic effect on the NB patient survival (Figure 1 and 2). In addition, CRT expression distinguished those with favorable outcomes from those with unfavorable outcomes in patients without N-myc amplification (Figure 3)

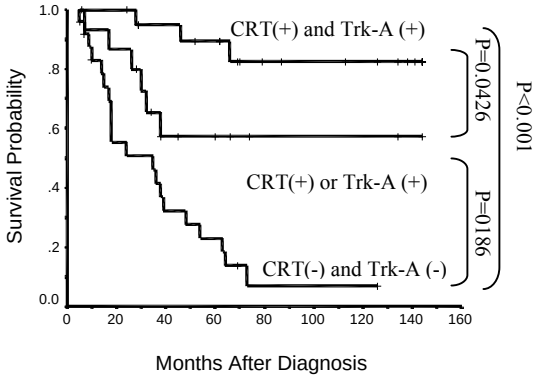

Figure 1. Cumulative survival curve according to the factors of CRT and Trk-A

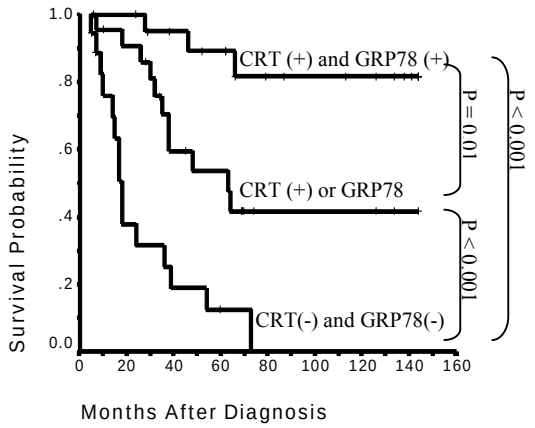

Figure 2. Cumulative survival curve according to the factors of CRT and GRP78.

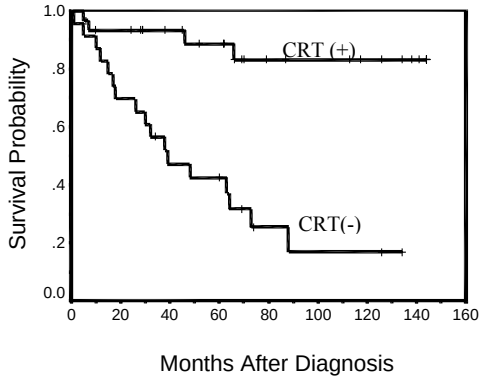

Figure 3. Cumulative survival curve according the factor of CRT in patients without N-*myc* amplification.

Discussion:

Favorable prognostic factors of NB are usually associated with the differentiation or death of the NB cells, such as Trk-A and Ras.[10,15] Interestingly, there are synergistic effects of these two factors on the NB cell death and the prognosis of this tumor,[16] reflecting both factors being involved in a common signaling pathway in the NB cells. NB cells expressing Trk-A, a nerve growth factor (NGF) receptor, may go to differentiation in the presence of NGF and death in its absence.[10] 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.[17] Activated PLC-γ1 acts to hydrolyze phosphatidylinositides to generate diacylglycerol, which may further activate the PKC-δ.[17] PKC-δ in turn is required for activation of the ERK cascade and for neurite outgrowth.[17] 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.[18] Thus, it is conceivable that CRT may participate in the process of Trk-A mediated NB cell differentiation and death just like Ras. Our study showed that CRT expression strongly correlated with the expression of Trk-A, in addition there was a synergistic effect of these two factors on patient survival. These evidence supports that CRT is involved in the Trk-A pathway.

All tumors in this study showed positive calnexin expression, suggesting that calnexin is required for all cells for survival. The differential expression of CRT was then not correlated with calnexin, which suggested that CRT played its role in NB probably not through its role of chaperone. On the contrary, CRT expression strongly correlated with GRP78 expression, in addition, both factors had synergistic effect on patient survival, suggesting that CRT might played its role in the differentiation of NB by its role as a stress protein as GRP78.

N-*myc* amplification is a well-known prognostic factor of NB.[13] Nevertheless only 22% (15/68) of patients were found to have tumors with N-*myc* amplification. In addition, since not all patients whose tumors had no N-*myc* amplification had very good prognoses, this category of patients was actually large groups of patients with clinical heterogeneity. Additional factors were required to further distinguish the prognoses of these patients. Our results showed that among patients with tumors without N-*myc* amplification, positive CRT expression distinguished the patients with favorable prognoses from those with unfavorable prognoses. These results indicate that immunohistochemical study of CRT may provide complementary prognostic information, which in turn may be helpful to the determination of the most appropriate intensity of therapy. In addition, the inverse correlationship between CRT and N-*myc* may also suggest that tumors with N-*myc* ampilification probably loss their ability to upregulate CRT and hence a rapid progressive behavior. It would be very intresting to further study the association between CRT and N-*myc*.

References

1. Michalak M, Corbett EF, Mesaeli N, et al: Calreticulin: one protein, one gene, many functions. Biochem J 344:281-292, 1999
2. Coppolino MG, Woodside MJ, Demaurex N, et al: Calreticulin is essential for integrin-mediated calcium signaling and cell adhesion. Nature 386:843-847,1997
3. Burns K, Duggan B, Atkinson EA, et al: Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature 367:476-480,1994.
4. Xiao G, Chung TF, Pyun HY, et al: KDEL proteins are found on the surface of NG 108-15 cells. Mol Brain Res 72:121-128,1999
5. Xiao G, Chung TF, Fine RE, et al: Calreticulin is transported to the surface of NG 108-15 cells where it forms surface patches and is partially degraded in an acidic compartment. J Neurosci Res 58:652-662,1999
6. Nakamura K, Bossy-Wetzel E, Burns K, et al: Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. J Cell Biol 150:731-740,2000

7. Rauch F, Prud'homme J, Arabian A, et al. Heart, brain, and body wall defects in mice lacking calreticulin. Exp Cell Res 2000; 256:105-111.
8. Nakamura K, Bossy-Wetzel E, Burns K, et al: Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. J Cell Biol 150:731-740, 2000
9. Liu H, Bowes RC, van de Water B, et al: Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca^{2+} disturbances, and cell death in renal epithelial cells. J Biol Chem 272:21751-21759,1997
10. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, et al: Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl Med 328:847-854, 1993
11. Joshi VV, Cantor AB, Altshuler G, et al. Recommendations for modification of terminology of neuroblastic tumors and prognostic significance of Shimada classification: a clinicopathologic study of 213 cases from the Pediatric Oncology Group. Cancer 1992; 69: 2183-2196.
12. Brodeur GM, Prichard J, Berthold F, et al. Revision of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993; 11: 1466-1477.
13. Castleberry RP: Pediatric update: neuroblastoma. Eur J Cancer 33:1430-1438,1997
14. Grady-Leopardi EF, Schwab M, Ablin AR, Rosenau W. Detection of N-myc oncogene expression in human neuroblastoma by in situ hybridization and blot analysis: relationship to clinical outcome. Cancer Res 1986;46:3196-9.
15. Kitanaka C, Kato K, Ijiri R, et al. Increased Ras expression and caspase-independent neuroblastoma cell death: possible mechanism of spontaneous neuroblastoma regression. J Natl Cancer Inst 2002; 94:358-368.
16. Tanaka T, Sugimoto T, Sawada T. Prognostic discrimination among neuroblastomas according to Ha-ras/trk A gene expression: a comparison of the profiles of neuroblastomas detected clinically and those detected through mass screening. Cancer 1998; 83:1626-1633.
17. Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001; 11: 272-280.
18. Rendón-Huerta E, Mendoza-Hernández G, Robles-Flores, M. Characterization of calreticulin as a protein interacting with protein kinase C. Biochem J 1999; 344: 469-475.