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Expressions of Rac1, Tiam1 and Cdc42 in retinoblastoma

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Abstract

The Rho GTPases are the molecular regulators of the cell motility processes and are involved in cell cycle progression and gene transcription. We studied the expression of Rho-like GTPases molecules, particularly Rac, Tiam1 and cdc42, in retinoblastoma and correlated these with clinicopathological parameters of the tumors. Sixty-seven tumors were included which were divided in to two groups; group A: tumors with optic nerve/choroidal/orbital invasion ($n = 35$) and group B: tumors with no invasion ($n = 32$). Immunohistochemistry was done on paraffin sections for all the proteins and were confirmed by Western blot on fresh tumor samples. In group A tumors, Rac was positive in 10/35 (28%), cdc42 was positive in 12/35 (34%) and Tiam1 was positive in 30/35 (85%) tumors. In group 2 tumors, Rac was positive in 5/32 (15%), cdc42 was positive in 4/32 (12%) and Tiam1 was positive in 30/32 (93%) tumors. Two groups (both invasive and non-invasive tumors) showed decreased expression of Rac1 and cdc42 whereas Tiam1 was significantly expressed in invasive tumors compared to non-invasive tumors ($P < 0.0001$). We observed a 70 K cleavage product of Tiam1 along with an 110 K product by blotting in RB samples. Caspase-3 was also demonstrated in RB samples, which showed Tiam1 cleavage products. This is the first study that showed the expression patterns of Rac, cdc42 and Tiam1 in retinoblastoma tumors. Thus, further studies are required to prove the involvement of caspase-3 in the cleavage of Tiam1 in vitro in RB cells and to trace out alternative pathways involved in tumor progression.

Keywords: retinoblastoma; invasion; Rac1; Tiam1; Cdc42

1. Introduction

Retinoblastoma is the most common intraocular malignancy in children. Factors that play a role in tumor invasion and metastasis are poorly understood. Aberrations in signaling pathways involved in the regulation of cell migration, cell–cell and cell–matrix interactions are known to contribute to tumor invasion and metastasis. While studies of modulators of tumor cell invasion such as matrix metalloproteinases (Surti et al., 2003; Mohan et al., 2005) and cadherins (Van Aken et al., 2002) have been performed in Retinoblastoma (RB), a common childhood eye tumor, there has not been much focus on downstream effectors of these processes.

Rho family members including Rac, Cdc42, and Rho are essential regulators of various cellular processes such as actin cytoskeleton reorganization, mitogenesis, activation of kinase cascades, transcriptional activation, and stimulation of DNA synthesis (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Teramoto et al., 2003). Interestingly, these small GTPases have been implicated in oncogenesis, through multiple approaches (Khosravi-Far et al., 1995; Olson et al., 1995; Zohn et al., 1998; Lin et al., 1999; Boettner and Van Aelst, 2002). Like all members of the Ras superfamily, Rho proteins function as molecular switches. They characteristically cycle between an active, GTP-bound state and an inactive, GDP-bound state. Switching between these states is mediated by guanine nucleotide exchange factors (GEFs), which promote the exchange

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of bound GDP for GTP and thereby activate the small GTPase (Van Aelst and D'Souza-Schorey, 1997). Tiam1 (T Lymphoma Invasion and Metastasis Suppressor) that belongs to Dbl family proteins is a Rac-specific GEF involved in the activation of small GTPase proteins (Rac, Cdc42, and Rho).

Earlier studies have shown that Rac and cdc42 proteins are potential stimulators of E2F-dependent transcription, capable of promoting hyperphosphorylation of retinoblastoma susceptibility gene product. There is no information available on the expression status of GTPase molecules and one of their activators Tiam1 in retinoblastoma.

The aim of the current study was to examine the expression of Rac1, Cdc42 and Tiam1 in retinoblastoma tumors and correlate our findings with clinicopathological parameters.

2. Materials and methods

2.1. Clinical information

Sixty-seven tumors were available from 67 eyes for the study. Among them, there were tumors from 39 males and 28 females. The age ranged from 1 month to 14 years (median = 33.45 months). There were 47 unilateral retinoblastomas, 18 bilateral retinoblastomas and 2 of unknown laterality.

2.2. Histopathological information

There were 32 tumors with no invasion of choroid or optic nerve and 35 tumors with invasion of chroroid/optic nerve/orbit. Among the 35 tumors with invasion, 8 had choroidal invasion alone (4 tumors with diffuse choroidal invasion and 4 with focal choroidal invasion). There were 26 tumors with invasion of optic nerve—19 with invasion of both choroid and optic nerve and 7 with invasion of optic nerve alone (2 with invasion of the prelaminar portion, 2 that had invaded up to lamina, 2 that had invaded to the post-laminar region and 1 that had invaded to the surgical end of the optic nerve head). There was a single tumor that had choroidal and orbital invasion. There were 20 well-differentiated tumors, 10 moderately differentiated tumors and 37 poorly differentiated tumors.

2.3. Antibodies

All primary antibodies were all purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, namely: Anti-human mouse monoclonal Cdc42 (B-8; 200 μg/ml) antibody, anti-human rabbit polyclonal Rac1 (C-14; 200 μg/ml) antibody, anti-human rabbit polyclonal T Lymphoma Invasion and Metastasis Suppressor (Tiam1) (H-300; 200 μg/ml) antibody and mouse monoclonal caspase-3 (E-8; 200 μg/ml) antibody.

2.4. Immunohistochemistry

In brief, paraffin sections (5 μm thick) were dewaxed and rehydrated. Antigen retrieval was performed by the pressure-cooker method in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 in H_2O (10 min) and the slides were incubated with the respective primary antibodies. Rac1 antibody was used at 1:50 dilution and incubated overnight, whereas Tiam1 and Cdc42 were used at 1:50 dilution for 2 h. Immunostaining was performed using Dako LSAB+ system horseradish peroxidase (Dakocytomation, Glostrup, Denmark). The reaction was revealed by 3,3′-diaminobenzidine tetrahydrochloride (Dakocytomation) and counterstained with hematoxylin. For negative control, the primary antibody was omitted and immunostain done.

2.5. Western blotting

Retinoblastoma tumor tissue (~250 mg) was collected from enucleated eyes ($n = 20$) and stored in phosphate-buffered saline (PBS, pH 7.0). All steps of this procedure were performed on ice. The samples were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.6), 5 mM EDTA, 150 mM sodium chloride, 0.1% phenylmethanesulfonylfluoride (PMSF, P7626, Sigma) and 250 μl of 1 mg/ml Proteinase Inhibitor Cocktail (P2714, Sigma) on ice. They were then passed through an 18-gauge needle, 3–5 times until all visible particles were dissolved. The samples were then centrifuged under cooling conditions at 5000 rpm (REMI C-24 Remi High Speed Cooling Centrifuge) until the supernatant was clear. For Western blotting of Rac1, the samples were further spun at 10,000 rpm for 30 min and centrifuged at 26,500 rpm for 4 h on a Beckman XL-80 Preparative Ultracentrifuge at 4 °C and the pellet fraction collected. Protein estimation was done by the method of Lowry et al. (1951).

Fifty micrograms of each sample was run on 12% SDS–PAGE gels and then electrophoretically transferred onto nitrocellulose membrane (Hybond ECL, Amersham) at 100 V for 2 h. Uniformity of transfer was checked by reversible staining of the membrane with 0.1% Ponceau S (Sigma) and non-specific sites were blocked with 10% non-fat dry milk for 90 min (Rac1), or 5% non-fat dry milk for 1 h (for Cdc42) or 10% non-fat dry milk for 2 h (Tiam1). The blots were incubated with primary antibody overnight at 4 °C in the following dilutions: Rac1 (1:1500), Tiam1 (1:1000) and Cdc42 (1:500). The blots were washed in three changes of TTBS (0.02 M Tris, pH 7.6 containing 0.1% Tween-20) and incubated with the respective HRP conjugated secondary antibody for 2 h at 1:10,00 (for Rac1, Cdc42) and 1:25,000 (for Tiam1) dilutions. After further washes with TTBS, the membranes were incubated with equal volumes of detection reagents A and B (Supersignal West Femto Maximum Sensitivity Substrate, Pierce).

2.6. Immunoanalysis

In each case, 20 randomly selected visual fields were examined under a microscope with a 20× objective lens and 10× oculars. In each visual field, 100 cells were examined, and cells expressing each antigen were counted. Thus the percentage of positive cells was calculated. Positivity was categorized as score 0 when no cells were stained, score 1 for 1–33% cells stained, score 2 for 34–66% cells stained and score 3 for >66% cells stained.