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Thymidylate Synthase 1 (TS1) in Situ Protein Expression Predicts the Survival of Ewing/PNET

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Abstract

TS1, RRM1 and ERCC1, which are crucial for DNA synthesis and repair and have shown prognostic and predictive value in carcinomas, were investigated in Ewing/PNET. The tissue microarray consisting of 31 archived Ewing/PNET samples was subjected to immunohistochemistry based on immunofluorescence combined with automated quantitative analysis (AQUA) to assess in situ expression. AQUA score was analyzed with various clinicopathological data. TS1 was immunoreactive in the nucleus and cytoplasm, while RRM1 and ERCC1 were nuclear. High TS1 expression, not RRM1 and ERCC1, was associated with long overall survival (p value = 0.057). Thus in situ TS1 protein expression in Ewing/PNET is a prognostic marker.

Keywords

Thymidylate synthase 1 (TS1); excision repair cross-complementation group 1(ERCC1); high ribonucleotide reductase M1 (RRM1); in situ protein expression; immunohistochemistry (IHC) based on immunofluorescence combined with automated quantitative analysis (AQUA); sarcoma; Ewing; PNET; tissue microarray (TMA)

Introduction

The pathogenesis and histogenesis of Ewing sarcoma/Primitive neuroectodermal tumor (PNET) is largely unknown. The prognosis of patients who develop advanced disease remains poor. Discovery of novel biomarkers that have prognostic and predictive values for this disease may shed light on better understanding of the patients' outcomes and guide the development of better therapeutic strategies. One of the approaches in searching for such biomarkers for Ewing sarcoma is to take advantage of current knowledge of known prognostic and predictive factors that have an impact on other malignancies.

As illustrated in figure 1, thymidylate synthase 1(TS1) is involved in deoxythymidine synthesis after conversion of uridine-diphosphate to deoxyuridine-diphosphate by

ribonucleotide reductase, and it is crucial for DNA synthesis and repair. TS1 protein expression is an independent prognostic factor of survival in colorectal cancer (ref 1), a predictor of response to 5-fluorouracil-based therapy for colorectal and gastric cancers (ref 2-3), and a clinically important determinant of survival in non-small cell lung cancer (NSCLC) (ref 4). RRM1, the regulatory subunit of ribonucleotide reductase, and ERCC1, a component of the 5'-nuclease involved in nucleotide excision repair, are also prognostic for the clinical outcome of NSCLC (Ref 5-7).

This study was intended to explore the role of the above biomarkers in Ewing/PNET by investigating the association between their protein expression patterns in archived tumor samples and the survival of corresponding patients.

Materials and Methods

This study was carried out in accordance with a research protocol approved by the Institutional Review Board at the Moffitt Cancer Center and the University of South Florida.

Tissue samples and patient data

A retrospective review was conducted to identify previously diagnosed Ewing/ PNET (1995-2007) archived at the Department of Anatomic Pathology of Moffitt Cancer Center. The diagnosis were verified by a sarcoma pathologist (MMB) following the criteria of Ewing sarcoma diagnosis accepted by the World Health Organization via histological examination of H&E slides and confirmation by immunohistochemical or molecular studies. The representative formalin fixed paraffin embedded tumor blocks were selected for tissue microarray (TMA) construction. The corresponding H&E slides of the TMA were reviewed to determine the tissue integrity prior to AQUA testing. TMA preparation was previously reported in this journal (ref 8).

Pertinent clinical data of these patients were compiled from two sources: 1) Pathology data base to include the patients' age, sex, tumor location, tumor size and ancillary study results. 2) Tumor registry to include the patients' treatment and survival information.

Immunofluorescence method

Immunofluorescence method used in this study was immunohistochemistry (IHC) based on immunofluorescence combined with automated quantitative analysis (AQUA). Immunofluorescence was used to assess in situ expression of the target molecules. Briefly, after being deparaffinized in xylene (3 × 10 min) and rehydrated through graded alcohols to tap water, the tissue array slides were treated with antigen retrieval solution for 5 min after boiling in a microwave oven, rinsed with wash buffer, treated with 0.3% H₂O₂ in water for 15 minutes, and then blocked for 30 min with 0.3% BSA. The slides were then incubated at -4 °C overnight with the first primary antibody (target antibody TS1, RRM1 and ERCC1 as in our previous reports (ref 4-7), and a second primary antibody CD 99, which was an antibody against human from mouse (Covance, Berkeley, CA) at 1: 100 dilution, or rabbit (abcom, Cambridge, MA) at 1:50 dilution. CD99 was used because it was consistently positive for Ewing sarcoma and therefore is an ideal label for Ewing cells. After washing, the slide was incubated with two different secondary antibodies for 1 hour (Envision® labeled polymer-HRP anti-rabbit or Envision® labeled polymer-HRP anti-mouse specific to the first primary antibody, and Alexa 555 goat-anti-mouse or goat-anti-rabbit, based on the source of second primary antibody. 1:200 dilutions in 0.3% BSA in buffer were used for both). Cy5-Tyramide (1:50 in Amplification solution) was added for ten minutes followed by mounting with Prolong Gold antifade reagent with DAPI mount solution. The slides were scanned with SpotGrabber and the image data were analyzed with AQUA which converts the

intensity of the fluorescent signal of the antibodies bound to the target antigens into quantitative digital data (PM-2000, HistoRx, New Haven, Conn).

Statistical analysis

The actual values for the AQUA scores of TS1, RRM1 and ERCC1 were treated as independent continuous variables and analyzed using the proportional hazards regression test. The primary objective was to assess the association between in situ protein expression of the above biomarkers and overall survival (OS) as well as disease free survival (DFS).

Results

Clinical and pathological data

Thirty one cases of Ewing/PNET were suitable for AQUA analysis. The patients' characteristics are shown in tables 1 and 2. Patients included 12 (38%) female, 19 (62%) male, mean age 37 years (range 12-72) and median size of tumor 7.0 cm (range 1.2-34.5). The most recent clinical outcome data show that 16 patients are alive and 15 dead with median follow-up 410 days. Ten patients (32.3%) were with local or distant metastatic disease.

Immunofluorescence result

TS1 was immunoreactive in the nucleus and cytoplasm, while RRM1 and ERCC1 were nuclear (figure 2), a pattern similar to that found in NSCLC (ref 4-7).

The prognostic values of TS1, RRM1 and ERCC1

Proportional hazard regression analysis revealed that high TS1 expression was associated with long overall survival (p value = 0.057) (table 3). RRM1 and ERCC1 expression level were not associated with survival. There was a marginal adverse effect of age, sex and tumor size on survival. Advanced age, female sex and tumor size were associated with shorter survival (table 4).

Discussion

Analysis of TS1, RRM1 and ERCC1 protein in Ewing/PNET has not been done to the best of our knowledge. Compared to standard IHC with visual scoring as a way to quantify protein expression, immunofluorescence offers an alternative in situ protein expression method that is objective and quantitative. Immunofluorescence provides continual, unimodal information for a spectrum of protein expression and overcomes the shortcoming of standard IHC including non-quantitative chemistry and subjectivity of light intensity perception by the pathologists (ref 4). In our hands, the immunofluorescence method has proven to be a simple, innovative and automated immunohistochemical method for the determination of the above biomarkers in archived Ewing sarcoma TMA samples. In addition, the subcellular location of the proteins was demonstrated. We found that TS1 was immunoreactive in the nucleus and cytoplasm, while RRM1 and ERCC1 were nuclear, a pattern similar to that found in NSCLC. One may suspect that the impact of TS1 expression on patients' outcomes may be associated with the subcellular compartment of its predominant localization. More importantly, we found that patients whose tumors expressed high levels of TS1 had a better outcome than those with low levels, which is similar to lung cancer; while RRM1 and ERCC1 expression levels were not associated with survival, which is in contrast to lung cancer. However, we are aware that the power of our analysis was limited by the size of sample.

Although studies with much larger sample sizes are warranted, potential mechanisms of TS1 as a potential prognostic marker may be speculated. Among genes involved in nucleotide synthesis needed for DNA damage repair, *TS1* is a particularly interesting candidate because of its gate keeper function for thymidine triphosphate (TTP) production. It is possible that tumors with efficient nucleotide metabolism and DNA repair pathways have an advantage over those with inefficient pathways with respect to ability to become resistant to DNA damaging chemotherapy. Although our study determined gene expression levels and not gene function, there is generally a direct relationship between TS1 protein levels and function, such as in lung cancers (ref 4). One way of testing of this hypothesis would be to see if there is any evidence of biologically significant posttranslational modification of TS1.

Conclusion

Similar to NSCLC, in situ TS1 protein expression in Ewing/PNET is a prognostic marker.

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References

1. Edler D, Kressener U, Ragnhammar P, et al. Immunohistochemically detected thymidylate synthase in colorectal cancer: an independent prognostic factor of survival. *Clin Cancer Res.* 2000; 6:488–492. [PubMed: 10690528]
2. Moertel CG, Fleming TR, McDonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med.* 1990; 322:352–358. [PubMed: 2300087]
3. Johnston PG, Lenz HJ, Leichman CG, et al. Thymidylate synthase gene and protein expression correlate and are associated with response to 9-fluorouracil in human colorectal and gastric tumors. *Cancer Res.* 1995; 55:1407–1412. [PubMed: 7882343]
4. Zheng Z, Li X, Schell MJ, Chen T, Boulware D, Robinson L, Sommers E, Bepler G. Thymidylate synthase in situ protein expression and survival in stage I nonsmall-cell lung cancer. *Cancer.* 2008; 112(12):2765–2773. [PubMed: 18442042]
5. Bepler G, Sharma S, Cantor A, et al. RRM1 and PTEN as prognostic parameters for overall and disease-free survival in patients with non-small-cell lung cancer. *J Clin Oncol.* 2004; 22:1878–1886. [PubMed: 15143080]
6. Simon GR, Sharma S, Cantor A, Smith P, Bepler G. ERCC1 expression is a predictor of survival in resected patients with non-small cell lung cancer. *Chest.* 2005; 127:978–983. [PubMed: 15764785]
7. Zheng Z, Chen T, Li X, Haura E, Sharma A, Pepler G. DNA synthesis and repair genes *RRM1* and *ERCC1* in lung cancer. *N Engl J Med.* 2007; 356(8):800–808. [PubMed: 17314339]
8. Schlauder S, Pledger J, Morgan M, Letson D, Ma Le, Bui MM. Assessment of muscarinic and nicotinic acetylcholine receptor (AChR) expression in primitive neuroectodermal tumor (PNET)/Ewing's family of tumor (EFT) and desmoplastic small round cell tumor (DSRCT): an immunohistochemical study of tissue microarray and western blot study of tumor cell lines. *Fetal and Pediatric Pathology.* 2008; 27:83–97. [PubMed: 18568996]

Fig. 1.

The methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is an essential step in the formation of thymine nucleotides.

a. Thymidylate synthase 1 (TS1) catalyzes this process by transferring the methyl base from 5,10-methylene-tetrahydrofolate (CH₂)-THF to dUMP, a key step for de novo DNA synthesis and cell proliferation.

b. TS1 is an important target for 5-fluorouracil (5FU). Its active metabolite, 5-fluoro-dUMP (FdUMP) forms a relatively stable ternary complex with TS1 and CH₂THF and inhibit TS1 function.

Fig. 2.

Representative of TS1, RRM1 and ERCC1 expression patterns in Ewing based on AQUA analysis. CD 99 stains in red as the tumor mask, nuclei stains in blue and target antigens stains in green. TS1 expression in Ewing tumor are located in both cytoplasm and nuclei, showing in green or orange/yellow after merged with CD 99. RRM1 and ERCC1 are both expressed in the nucleus only.

Table 1

The pertinent pathological and clinical data

Case	Sex	Age	Location	Tumor Size (cm)	Metastasis *
1	M	24	Chest wall	10.5	No
2	M	17	Lower lobe lung	21.5	No
3	F	72	Thigh	6	No
4	M	28	Pelvis	14	Yes
5	M	30	Thigh	17	No
6	F	28	Femur	12	Yes
7	M	40	Pelvis	10	No
8	M	17	Pelvis	N/A	Yes
9	M	24	Pelvis	12.7	No
10	M	59	Thigh	4.5	Yes
11	F	15	Shoulder/Humerus	5.3	No
12	M	33	Spine (T7)	1.6	Yes
13	F	15	Femur	3.7	Yes
14	F	57	Leg	34.5	No
15	M	12	Distal femur	11	No
16	M	35	Flank	3	No
17	M	58	Thigh	8	Yes
18	F	35	Fibula	7	No
19	M	54	Chest	8	No
20	M	15	Ilium	7.5	No
21	M	28	Rib	5	No
22	F	61	Chest	7.5	No
23	M	16	Pelvis	7.5	No
24	F	16	Rib	5	No
25	M	14	Leg	N/A	No
26	F	71	Uterus	11	Yes
27	M	29	Thigh	30	No
28	F	41	Lung	8	Yes
29	M	34	Chest wall	5.7	No
30	F	67	Lung	7	Yes
31	F	20	Brain	1.2	No

* Metastasis at diagnosis or follow-up

Table 2

The pertinent pathological and clinical data continued

Case	Treatment	OS (days)	DFS (days)	Vital Status
1	S, C, R	610	323	Dead
2	C, R	623	309	Dead
3	S, C, R	298	157	Dead
4	C, R	22	95	Dead
5	S, C, R	2743	2652	Alive
6	S,C, R	522	417	Alive
7	S, C, R	1700	1705	Alive
8	C, R	1029	615	Dead
9	S, C, R	1067	887	Alive
10	S, C	1624	1301	Alive
11	S, C	4036	3914	Alive
12	S, C, R	209	163	Dead
13	S, C	710	710	Dead
14	S, C	506	311	Dead
15	S, C, R	5065	4224	Alive
16	S, C, R	3892	3815	Alive
17	S, C, R	661	491	Alive
18	S, C, R	1301	1083	Alive
19	S, C, R	681	681	Dead
20	S, C, R	1829	977	Dead
21	S, C, R	1553	1302	Dead
22	S, C	364	197	Dead
23	S, C, R	1929	615	Alive
24	S, C	260	2233	Alive
25	S, C	637	637	Alive
26	S	25	25	Dead
27	S, C, R	2743	2652	Alive
28	S, C, R	2388	1883	Dead
29	S, C	260	2253	Alive
30	S, C, R	341	319	Dead
31	S, C,R	806	687	Alive

S: Surgery; C: Chemotherapy; R: Radiation

Table 3

Proportional hazard regression analysis of TS1 expression and survival

Table 1	Hazard Ratio	P-value	95% confidence interval
OS	0.021	0.0573	0.000, 1.1298
DFS	0.067	0.1764	0.001, 3.381

Table 4

Proportional hazard regression analysis of age, sex and tumor size on survival

Variable	Outcome	Hazard Ratio	P-Value	95% C.I.
Age	OS	1.028	0.0779	(0.997, 1.061)
Age	DFS	1.026	0.1203	(0.993, 1.059)
Sex (M:F)	OS	0.398	0.0135	(0.192, 0.827)
Sex (M:F)	DFS	0.475	0.0431	(0.231, 0.977)
Tumor Size	OS	1.207	0.0217	(1.028, 1.418)
Tumor Size	DFS	1.077	0.0639	(0.996, 1.165)