International Orthopaedics (SICOT) (2004) 28:222–225
DOI 10.1007/s00264-004-0551-4

ORIGINAL PAPER

J. G. Grohs · A. Zoubek · D. Jugovic · H. Kovar · R. Windhager

Intraoperative dissemination of tumour cells in patients with Ewing tumours detected by RT-PCR

Accepted: 18 February 2004 / Published online: 16 March 2004

Abstract Using reverse transcriptase polymerase chain reaction (RT-PCR) we evaluated the occurrence of tumour-cell ribonucleic acid (RNA) in the blood during surgery in patients with Ewing tumours. The patients received irradiation and chemotherapy according to the protocol of the European Intergroup Cooperative Ewing Sarcoma Study (EICESS) 92. Blood samples were taken from 15 patients. Intra-operative dissemination was found during 2/8 resections but showed no relation to patient survival. At second-look biopsy, detection of tumour-cell RNA was associated with relapse and metastases in 3/4 patients. The results suggest that pre-operative treatment did not completely prevent dissemination of tumour cells during surgery of Ewing tumours.

Résumé En utilisant le dosage par réaction de polymérase (PCR) de la transcriptase inverse nous avons évalué la présence de l'acide ribonucléique cellulaire (ARN) tumoral dans le sang pendant la chirurgie pour tumeur d'Ewing. Les malades ont reçu radiothérapie et chimiothérapie d'après le protocole de l'Intergroupe européen Ewing Sarcome Étude Coopérative (EICESS) 92. Des échantillons de sang ont été prélevés chez 15 malades. La dissémination intraopératoire a été trouvée pendant 2 résections sur 8, mais n'a pas montré de relation avec la survie des malades. À la biopsie de contrôle la détection d'ARN tumoral était associée à la rechute et les métastases chez 3/4 des malades. Les résultats suggèrent que le traitement pré-opératoire n'a pas complètement prévenu la dissémination de cellules tumorales pendant la chirurgie des tumeurs d'Ewing.

J. G. Grohs (✉)
Department of Orthopaedic Surgery,
University of Vienna,
Währinger Gürtel 18–20, 1090 Wien, Austria
e-mail: josef.grohs@meduniwien.ac.at
Tel.: +43-1-40400-4078
Fax: +43-1-40400-4077

A. Zoubek · D. Jugovic · H. Kovar
CCRI, St Anna Children Hospital,
Vienna, Austria

R. Windhager
Department of Orthopaedic Surgery,
University of Graz,
Austria

Introduction

Without prior chemotherapy, manipulation of primary localised malignant tumours has been shown to induce shedding of tumour cells into the bloodstream during surgery [1, 4, 8, 9]. The reverse transcription polymerase chain reaction (RT-PCR) was used for detection of tumour cells during surgery of breast cancer [1], colorectal cancer [4], prostate cancer [8], and Ewing's sarcoma in blood samples from peripheral veins [17]. Tumour cells from peripheral veins of patients with breast cancer were viable [12]. The implantation of circulating tumour cells was minimised by host defences [6] in humans. Nevertheless, dissemination of tumour cells during surgery might support the development of metastases.

In patients with localised Ewing's sarcoma, systemic relapses of the disease have occurred despite good response to chemotherapy and irradiation [7] with less than 10% vital tumour. It is still a matter of speculation whether these treatment failures can be attributed to the shedding of tumour cells during resection. Therefore, it is of interest whether pre-operative systemic and local treatment has any protective effect against the dissemination of tumour cells during surgery. Ewing tumours are well characterised by a rearrangement of genes [3]. The presence of tumour-specific chimeric transcripts resulting from the fusion of these gene was used for high-sensitivity detection of tumour cells in blood and bone marrow by the method of RT-PCR [11, 18, 15, 16].

The aim of the present study was to document tumour-cell dissemination into the blood by detection of tumour-cell ribonucleic acid (RNA) during open biopsy before treatment, during second-look biopsy, or tumour resection after chemotherapy and irradiation.

Patients and methods

Fifteen patients at an age of 1.7–29 years (Table 1) were treated according to the protocol of the European Intergroup Cooperative Ewing Sarcoma Study (EICESS) 92. Treatment included chemotherapy with etoposide, vincristine, actinomycin D, ifosfamide and adriaymcin; irradiation and, if feasible, tumour resection. The tumours were classified histologically as Ewing tumours. Twelve of 15 patients had localised disease, two had synchronic lung metastases and one was a local relapse. Seven patients were studied at the time of biopsy before systemic treatment, eight during resection of the tumour and four during second-look biopsy after chemotherapy and local irradiation (Table 2). Second-look biopsy was performed to confirm necrosis after chemotherapy and irradiation of inoperable tumours. Histological tumour response to therapy (Table 2) was graded according to the criteria published by Salzer-Kuntschik [13] and adapted for Ewing tumours by Jürgens [7]. No decisions relevant to the treatment of the respective patients were drawn from the results of this study.

Tumour fragments from the primary tumour site were immediately snap-frozen in liquid nitrogen for characterisation of the fusion transcript type of the individual tumour (Table 1). At biopsies and second-look surgeries, blood samples were collected from the peripheral venous blood through a fixed intravenous canula after onset of anaesthesia before incision of the skin and during skin closure. At resections, the samples were taken before skin incision, during tumour preparation and during skin closure. Additional samples were collected 2 days following surgery. Further samples were collected directly from the lowest spot of the operation field after opening of the bone or resection of the tumour, respectively. After red blood cell lysis followed by centrifugation, total RNA was isolated using the acid-guanidinium-phenol/chloroform method [2]. The resulting desoxyribonucleic acids (cDNAs) were PCR amplified under previously published conditions using adequate primers [3, 18]. In order to minimise the risk of sample-to-sample contamination, a one-tube-nested RT-PCR protocol was applied and additional positive and negative controls were used as previously described [10]. The sensitivity of this method allows the detection of one tumour cell in 1×10^6 nucleated peripheral blood cells [11, 18].

Results

During biopsy (patients 1, 2, 3, 4, 11, 13 and14), all samples from the operation site contained tumour-cell RNA. In the peripheral blood, tumour-cell RNA was

Table 1 Data of patients and tumours. *M* male, *F* female, *ex* exon, *NED* no evidence of disease, *DOD* died of disease, *DOC* died of complication, *POD* progress of disease

Patient	Age	Gender	Localisation	Histology	Fusion type	Stage at diagnosis	Follow-up
1	14	F	Ilium	Ewing	EWS ex 7 / FLI-1 ex 6	Lung metastases	DOD
2	14	F	Ilium	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	NED
3	0 8	F	Pubis	Ewing	EWS ex 7 / FLI-1 ex 5	Localised	NED
4	26	F	Ilium	PPNET	EWS ex 7 / FLI-1 ex 6	Localised	DOC
5	1.7	M	Ilium	Ewing	EWS ex 7 / FLI-1 ex 5	Localised	NED
6	29	F	Ilium	PPNET	EWS ex 7 / FLI-1 ex 6	Localised	POD
7	24	M	Sacrum	Ewing	EWS ex 7 / FLI-1 ex 6	Local relapse	DOD
8	27	M	Ilium	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	DOD
9	0 4	M	Femur	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	NED
10	10	F	Ilium	Ewing	EWS ex 7 / FLI-1 ex 6	Lung metastases	NED
11	0 8	F	Femur	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	POD
12	17	M	Tibia	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	NED
13	16	M	Fibula	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	NED
14	19	F	Ilium	Ewing	EWS ex 7 / FLI-1 ex 6	Localised	DOD
15	27	F	Femur	Atypical Ewing	EWS ex 7 / FLI-1 ex 6	Localised	NED

Table 2 Surgery, tumour response to treatment, and samples containing tumour-cell ribonucleic acid (RNA). *Nb,r* first and second surgery of one patient. Degree of regression according to Salzer-Kuntschik et al (1987). *RTX* irradiation, *OP site* operation site, *(E)VAIA* etoposide, vincristine, actinomycin D, ifosfamide, adriamycin. Sample with (+) and without (-) tumour-cell RNA, sample without tumour-cell RNA, but not all quality criteria fulfilled (-?), *Md* missing data

Patient	Surgery	Chemotherapy cycles/RTX before surgery	Tumour regression (degree)	Peripheral blood			OP site
				Before surgery	Before osteotomy	After surgery	
5b	Open second look	14 EVAIA, 45 Gy	3	-		+	Md
6	Open second look	7 EVAIA, 45 Gy	No tumour cells	md		+	+
7b	Open second look	13 EVAIA, 56 Gy	No tumour cells	+		+	+
8	Open second look	7 EVAIA, 45 Gy	no tumour cells	+		-	-
9	Wide resection	5 EVAIA, 54 Gy	1	-	-	+	+
10	Wide resection	8 EVAIA, 54 Gy	1	-	-	+	+
5r	Wide resection	14 EVAIA, 45 Gy + 2 high dose cycles	1	-	-	-	-
3r	Wide resection	7 VAIA, 45 Gy	1	-?	-	-	-?
4r	Radical resection	7 VAIA, 45 Gy	4	-	-	-?	-
11r	Wide resection	8 EVAIA	3	-	-	-	-
12	Wide resection	4 VAIA	1	-	-	-	-
15	Wide resection	3 EVAIA	1	-	-	-	-

detected in 1/7 samples before biopsy (patient 13) and in another one after biopsy (patient 2).

Before second-look biopsy, the venous blood of 2/4 samples contained tumour-cell RNA. In two further patients, the peripheral blood turned positive during surgery. At the operation site, tumour-cell RNA was found in 2/4 samples. In none of the eight samples was tumour-cell RNA found in the peripheral blood before resection. At the operation site, tumour-cell RNA was detected in 2/8 samples 30 and 60 min after skin incision, respectively. At the end of surgery, tumour-cell RNA was detectable also in the peripheral blood of these two patients.

Clearance of tumour-cell RNA from peripheral blood was investigated in four patients. One patient still had tumour-cell RNA in the peripheral blood after 42 h, although she had received chemotherapy during this time. At definitive surgery, the tumour had to be classified as a non-responder. The median follow-up period was 3.1 (range 0.5–6.4 years). Nine months after tumour resection, one patient with histologically poor response to therapy had a local relapse. Two patients had a local relapse and one patient a systemic relapse 7 (range 5.5 – 53 months) after second-look biopsy. In two patients (Table 2, patients 7 and 8) who had received only chemotherapy and irradiation, no vital tumour cells could be detected by conventional histology at second-look biopsy. Nevertheless, these patients were the only ones with RNA-positive samples in peripheral blood.

Discussion

Several investigations deal with the prognostic factors of Ewing tumours [7, 10]. Intra-operative dissemination of tumour cells may have been a reason for relapse in about 10% of Ewing tumours, despite good prognostic features such as small volume, good response to therapy and wide resection margins [7]. RT-PCR is a very sensitive and specific tool for detection of Ewing tumour cells. The detection of tumour-cell RNA at the operation site and peripheral blood confirm recent studies that described dissemination of carcinoma cells to peripheral blood [1, 4, 9]. Furthermore, the present study describes intra-operative mobilisation of tumour cells as evidenced by detection of RNA after chemotherapy and irradiation for the first time.

In other studies, the viability of circulating breast cancer cells was proven by colony growth [12]. In our investigation, RNA was detected in peripheral blood up to 42 h after surgery in a patient with a non-responding tumour. From these results, it can be deduced that we detected not only RNA but whole cells as well, since cell fragments and RNA should be cleared from blood within a few hours.

Detection of tumour-cell RNA at the operation site and in circulating blood during intra-lesional second-look biopsy indicates poor tumour response to local or systemic therapy, even if no tumour is found in the specimen by conventional histology. This observation might lead to the implementation of PCR in the course of diagnostics. Circulating tumour cells were dependent on the tumour volume [14]. Nevertheless, only the presence of tumour cells in the bone marrow but not in blood was associated with an adverse prognosis [5, 14]. The performance of second-look biopsies possibly should be discussed under the aspect that highly resistant tumour cells could be distributed. Especially under the conditions of patients having finished the maximum chemotherapy, we do not have sufficient means for further therapy. It remains to be verified whether circulating tumour cells arise from micro-metastatic sites or the local relapse [14].

In the present study, we detected Ewing tumour-cell RNA at the operation site during wide resection of tumours that showed an excellent response to therapy. Detection of tumour-cell RNA showed no relation to the histological response to therapy. Both patients with RNA-positive samples during resection survived 4.3 and 6.3 years without any evidence of disease. It has to be supposed that either dissemination of these tumour cells into the circulation did not result in metastases [6] or post-operative chemotherapy prevented a relapse.

Despite good response to pre-operative treatment, tumour cells survive until surgery. The detection of tumour-cell RNA in peripheral blood before or during second-look biopsy might be useful to modify the time course and doses of post-operative chemotherapy in these un-resectable tumours.

Acknowledgement This work was supported by a grant of Jubiläumsfond of the Österreichische Nationalbank (Grant no: 4893) to HK and by private donations to the CCRI. We thank our pathologist Dr. G. Amann for the histological investigations.

References

1. Brown DC, Purushotam AD, Birnie GD, George WD (1996) Detection of intraoperative tumor cell dissemination in patients with breast cancer by use of reverse transcription and polymerase chain reaction. Surgery 117:96–101
2. Chomoczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. Anal Biochem 162:156–159
3. Delattre O, Zucman J, Melot T, Garau XS, Zucker JM, Lenoir G, Ambros PF, Sheer D, Turc-Carel C, Triche T, Aurias A, Thomas G (1994) The Ewing family of tumors—A subgroup of small-round-cell tumors defined by specific chimeric transcripts. N Engl J Med 331:294–299
4. Dobrovic A, Hardingham A, Kotasek D, Eaton MC, Mi JX, Sage RE (1995) Detection of circulating tumor cells pre- and post- surgery for colorectal cancer using immunobead-PCR. Proc Am Assoc Cancer Res 36:214
5. Fagnou C, Michon J, Peter M (1998) Presence of tumor cells in bone marrow but not in blood is associated with adverse prognosis in patients with Ewing's tumor. J Clin Oncol 16:1707–1711
6. Fidler I (1991) The biology of human cancer Metastasis. Acta Oncol 30:669–675
7. Jürgens H, Exner U, Gadner H, Harms D, Michaelis J, Sauer R, Treuner J, Voute T, Winkelmann W, Winkler K, Göbel U (1988) Multidisciplinary treatment of primary Ewing's sarcoma of bone. Cancer 61:23–31
8. Katz AE, Olsson CA, Raffo AJ, Cama C, Perlman H, Seaman E, O'Toole K, McMahon D, Benson M, Buttyan R (1994)

Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. Urology 43:765–774
9. Mori M, Mimory K, Ueo H, Karimine N, Bernhard G, Sugimachi K, Akiyoshi T (1996) Molecular detection of circulating solid carcinoma cells in the peripheral blood: the concept of early systemic disease. Int J Cancer 68:739–743
10. Peter M, Magdelenat H, Michon J, Melot T, Oberlin O, Zucker JM, Thomas G, Delattre O (1995) Sensitiv detection of occult Ewing's cells by reverse transcriptase-polymerase chain reaction. Br J Cancer 72:96–100
11. Pfleiderer C, Zoubek A, Gruber B, Kronberger M, Ambros PF, Lion T, Fink FM, Gadner H, Kovar H (1995) Detection of tumor cells in peripheral blood and bone marrow from Ewing tumor patients by RT-PCR. Int J Cancer 64:135–139
12. Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D, Winter C, Akard L, Jansen J, Copelan E, Meagher RC, Herzig RH, Klumpp TR, Kahn DG, Warner NE (1993) Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. Blood 82:2605–2610
13. Salzer-Kuntschik M, Delling D, Beron G, Sigmund R (1983) Morphological grades of regression on osteosarcoma after polychemotherapy: Study COSS 80. J Cancer Res Clin Oncol 106 [Suppl]:21–24
14. Schleiermacher G, Peter M, Oberlin O, Philip T, Rubie H, Mechinaud F, Sommelet-Olive D, Landman-Parker J, Bours D, Michon J, Delattre O (2003) Increased risk of systemic relapses associated with bone marrow micrometastasis and circulating tumor cells in localized Ewing Tumor. J Clin Oncol 21:85–91
15. Sorensen PHB, Liu XF, Delattre O, Roeland JM, Biggs CA, Thomas G, Triche TJ (1993) Reverse transcriptase PCR amplification of EWS/FLI-1 fusion transcripts as a diagnostic test for peripheral primitive neuroectodermal tumors of childhood. Diag Molec Pathol 2:147–57
16. Zoubek A, Pfleiderer C, Salzer-Kuntschik M, Amann G, Windhager R, Fink FM, Koscielniak E, Delattre O, Strehl S, Ambros PF, Gadner H, Kovar H (1994) Variability of EWS chimaeric transcripts in Ewing tumours: a comparison of clinical and molecular data. Br J Cancer 70:908–913
17. Zoubek A, Kovar H, Gruber B, Kronberger M, Windhager R, Amann G, Gadner H (1996) Mobilization of tumor cells during biopsy in an infant with Ewing's sarcoma. Eur J Pediatr 155:373–376
18. Zoubek A, Ladenstein R, Windhager R, Amann G, Fischmeister G, Kager L, Jugovic D, Ambros PF, Gadner H, Kovar H (1998) Predictive potential of testing for bone marrow involvement in Ewing tumor patients by RT-PCR: a preliminary evaluation. Int J Cancer 79:56–60