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Eur J Cancer, Vol. 28A, No. 8/9, pp. 1441–1446, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Expression of Glutathione-S-transferase-π in Human Tumours

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Expression of glutathione-S-transferase-π (GST-π) gene was quantitatively analysed on various human tumours (renal cell, colorectal, head and neck, ovarian carcinomas, soft tissue sarcomas; non-Hodgkin lymphomas) and on the corresponding normal tissues when available (kidney, colorectum and head and neck). GST-π mRNA expression level was found to be significantly higher in tumours ($P<0.01$) than in the normal counterparts (mainly 7.3-, 3.5- and 3.0-fold in colorectal, head and neck, and renal carcinomas, respectively). Most tumours displayed a significant relationship between higher GST-π expression level and poor differentiation grade of tumour cells, thus suggesting a relationship between GST-π activity, neoplastic transformation and cellular differentiation grade. The high requirement of GST-π activity neoplastic cells displayed was not singularly related to cellular replication rate. Finally, GST-π gene expression levels were not affected by chemotherapeutic treatments.
Eur J Cancer, Vol. 28A, No. 8/9, pp. 1441–1446, 1992.

INTRODUCION

GLUTATHIONE-S-TRANSFERASE (GST) is a family of multifunctional enzymes which catalyse the nucleophilic addition of glutathione to a wide heterogeneous groups of compounds [1]. A variety of biological functions have been ascribed to this isozyme group, including intracellular binding and transport of lipophilic compounds such as bile products, steroid hormones, drugs and other xenobiotics [2]. By far the most studied function of GST enzymes is their role in cellular detoxification, primarily against oxygen-free radicals and peroxides, produced by cellular physiological processes and exogenous stimuli [1,3].

An anionic isozyme class of GST has attracted strong interest in the oncological field because of its ubiquitous and quantitatively high expression in chemically induced tumours [4, 5] and in cell lines transformed by transfected viral oncogenes [6]. The human isozyme of this class, GST-π, was found to be expressed in elevated amounts in most tumours belonging to almost all histological types [7–10]. These findings have suggested that the biological action of GST-π is of considerable importance to neoplastic cell survival. Moreover, the frequent quantitative increase of GST-π in drug-resistant cell lines, sometimes associated with the enhanced expression of the MDR1 gene product [6,11–13], seems to indicate a possible link between GST-π and the multidrug-resistant (MDR) phenotype.

To ascertain a possible functional association of GST-π overexpression with neoplastic transformation and/or cellular drug resistance, we studied a series of human tumours [renal cell, colorectum, head and neck, ovarian carcinomas, soft tissue sarcomas; non-Hodgkin malignant lymphomas (NHL)] and their corresponding normal tissues, when available (kidney, colorectum, head and neck).

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Revised 29 Jan. 1992; accepted 14 Feb. 1992.

Table 1. Cancer types and most frequent tumour histotypes

Cancer type	No.	Histotype	n
Kidney	29	Renal cell adenocarcinoma	29
Colorectum	29	Adenocarcinoma	29
Head and neck	14	Squamous cell carcinoma	13
Ovary	24	Adenocarcinoma	8
		Papillary adenocarcinoma	7
		Papillary cystoadenocarcinoma	5
		Endometroid adenocarcinoma	3
Soft tissue	36	Malignant fibrous histiocytoma	14
		Liposarcoma	6
		Synovial sarcoma	4
Lymphoid tissue	18	Low grade B-cell NHL	9
		High grade B-cell NHL	9

Tumour samples were obtained from 126 chemotherapy-untreated patients and 24 patients pretreated with drugs, including doxorubicin and/or cisplatin involved in drug resistance mediated by GST-π [14].

MATERIALS AND METHODS

Tumour Samples

Tumour biopsy specimens, cleared of necrotic and haemorragic tissues, were frozen in liquid nitrogen immediately after surgical removal and stored at −80°C. Routine histology confirmed that the samples consisted of tumour cells. Staging and histological grading were based on the American Joint Committee on Cancer Criteria [15]. Matched normal tissues were obtained from the surgical margins sited as far as possible from the tumour mass. For head and neck, and colorectal normal tissues, in order to avoid large contamination with tissues not corresponding with the tumoural histotype, mucosal layer was deprived as much as possible of connective tissue and muscle. Normal tissues were stored at −80°C. The age of the 150 patients (68 men and 82 women), ranged from 18 to 82 years (mean 59.5). Tumour histotypes are shown in Table 1.

126 primary tumours from untreated and 24 tumoral specimens from chemotherapeutically pretreated patients were obtained at surgery. The 12 pretreated soft tissue sarcomas were part of a clinical trial on the efficacy of an intra-arterial presurgical chemotherapy with doxorubicin and iphosphamide (intravenous) in high grade (G3) primary sarcomas localised to the limbs (3 patients showed a response < 50%, 2 were not changed and 4 were in progression). The 12 pretreated ovarian cancers (3 primary and 9 metastatic — FIGO stage III and stage IV) received various chemotherapeutic regimens all including cisplatin, cyclophosphamide and doxorubicin. All these patients exhibited macroscopic residual tumour masses larger than 2 cm and were judged as poor responders to chemotherapy.

Cell lines

DLD1, SW948 and SW1417 human colon carcinoma cell lines were obtained from the American Type Culture Collection (Rockville). Cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum from Seralab (Sussex), 1 mmol/l sodium pyruvate and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air.

RNA extraction, northern and dot blot analyses

Frozen tissues were pulverised by a microdismembrator and total cellular RNA was extracted by the guanidine chloride method [16]. Cultured cells were directly lysed in the guanidine chloride solution. For northern blot analysis, 10 μg total RNA were fractionated by electrophoresis in a denaturing 1% agarose/6% formaldehyde agarose gel and transferred to Gene Screen Plus membrane (New England Nuclear) by electroblotting in 0.025 mol/l phosphate buffer pH 6.5 (10 V overnight and 40 V for 1 h).

For dot blot analysis, Gene Screen Plus membrane was presoaked in distilled water then 1−, 3− and 10− μg aliquots of denatured RNA were loaded using a BRL hybri-dot Manifold apparatus (BRL, Gaithersburg, Maryland). Membranes were prehybridised and hybridised [17] and exposed to X-ray films with an intensifying screen at −80°C. Hybridisation signals were quantified by densitometric scanning. Quantitation of GST-π, histone H3 and MDR1 mRNA expression levels was performed on total RNA preparations which showed undegraded ribosomal RNA at the agarose gel electrophoresis and revealed a single band dimensionally coherent with the size of the specific mRNA at the northern blot. β-Actin mRNA level was used as internal standard. GST-π, histone H3 and MDR1 mRNA expression levels are reported in arbitrary expression units (EU). Values of 10 EU for MDR1 mRNA, 100 EU for GST-π mRNA and 100 EU for histone H3 were assigned to the hybridisation signals produced by 10 μg of a single preparation of total RNA extracted from DLD1 human colon carcinoma cell line.

Southern blot analysis

High molecular weight DNA was extracted from frozen specimens by the phenol method [18]. Samples of 10 μg of DNA were digested with restriction enzymes, electrophoresed in agarose gel (1%) and transferred on to Gene Screen Plus membrane, by the procedure of Southern [19]. Membranes were prehybridised, hybridised and washed as described [17].

Probes

Probes used were: 0.75 kb *Eco*RI fragment derived from plasmid pGPi2 [20] specific for GST-π gene; 2.1 kb *Eco*RI fragment derived from plasmid PF0422 [21] specific for histone H3 gene; 1.2 kb *Eco*RI fragment derived from plasmid pHuP170#1 [22] specific for MDR1 gene; 0.45 kb *Hind*II–PstI fragment derived from plasmid HepG2-16 [23] specific for MDR 2/3 gene and the 0.7 kb *Eco*RI–*Bam*HI fragment derived from plasmid pHF βa-3′ UT [24] specific for β-actin gene.

Probes were labelled with ^{32}P using a multiprime labelling system (Amersham) at specific activity $>10^9$ cpm/μg DNA.

Statistics

The variables under study were not normally distributed, therefore, nonparametric statistics were chosen. Mann–Whitney test was used to compare the distribution of GST-π and MDR1 expression levels between 2 groups: e.g. untreated vs. treated tumours. Wilcoxon test was used to compare the distribution between 2 paired groups: e.g. GST-π in normal and neoplastic tissues. The Kruskall–Wallis test was used to compare the expression of GST-π, histone H3 or MDR1 mRNAs, between 3 groups: e.g. G1, G2 and G3 grade. Pearson product–moment correlations test was used to ascertain the correlation between continuous variables: e.g. GST-π and histone H3 levels.

Results were considered as statistically significant at $P \leq 0.05$.

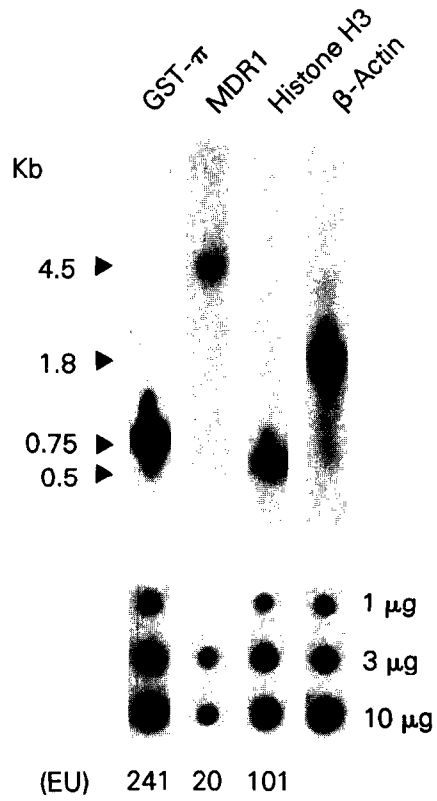

Fig. 1. Northern blot (upper) and dot blot (lower) mRNA expression analysis of GST-π, MDR1, histone H3 and β-actin. A representative colorectal carcinoma sample is shown. mRNA sizes are indicated on the left. EU, expression units.

RESULTS

GST-π mRNA expression

GST-π mRNA expression level was determined by dot blot hybridisation analysis on undegraded RNA samples. Specificity of the dot blot hybridisation was confirmed by northern blot analysis which constantly detected a single 0.75 kb hybridisation signal dimensionally coherent with the GST-π mRNA. Densitometric values corrected for β-actin hybridisation signal were reported as arbitrary expression units (EU) (see Materials and Methods) (Fig. 1). The range of GST-π mRNA expression level in tumour samples varied from undetectable to 452 EU [mean (S.D.) 126.55 (94.76), median 97 EU] (Fig. 2). On average, ovarian, colorectal, head and neck carcinomas and soft tissue sarcomas expressed higher levels of GST-π mRNA than renal cell carcinomas and NHL (Fig. 2). High intertumour variability in GST-π mRNA expression level was observed in each tumour group (Fig.2). On the basis of the histological analysis of tissue samples contiguous to those used for RNA extraction, we can reasonably exclude that the intertumour variability in GST-π mRNA expression level was consequent upon large contamination with non-neoplastic cells or necrosis. Such variability was possibly due to differential mRNA gene level since neither gene amplification nor rearrangements were detected, by *Eco*RI, *Bam*HI and *Hind*III digestion and Southern blot analysis (data not shown).

Among clinical and pathological parameters considered (patient age, sex, clinical stage, tumour histotype and tumour grading), only tumour grading showed a significant correlation with GST-π expression level in renal cell carcinomas ($P < 0.01$), soft tissue sarcomas ($P = 0.01$), ovarian carcinomas ($P < 0.05$) and NHL lymphomas ($P < 0.05$) (Fig. 3). On the contrary, no

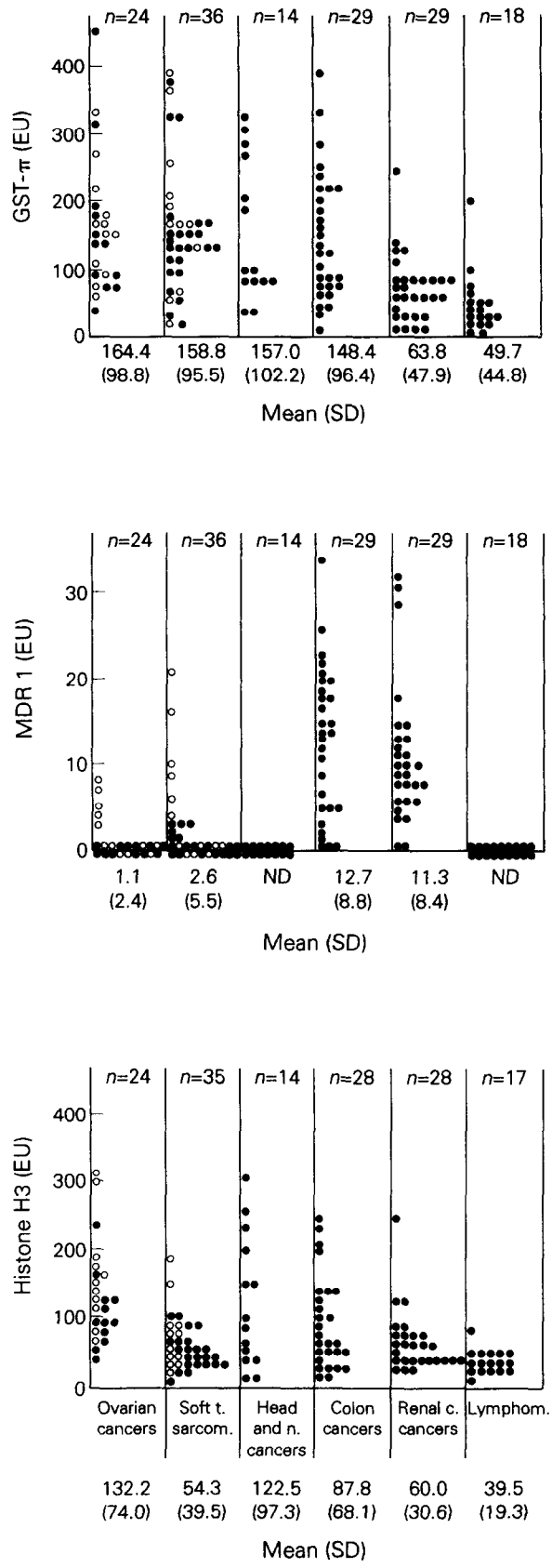

Fig. 2. mRNA expression levels in tumour samples of different types. Results obtained by hybridisation with GST-π, MRD1 and histone H3 probes are graphically displayed. EU, expression units. (○) Chemotherapeutically pretreated patients and (●) untreated patients.

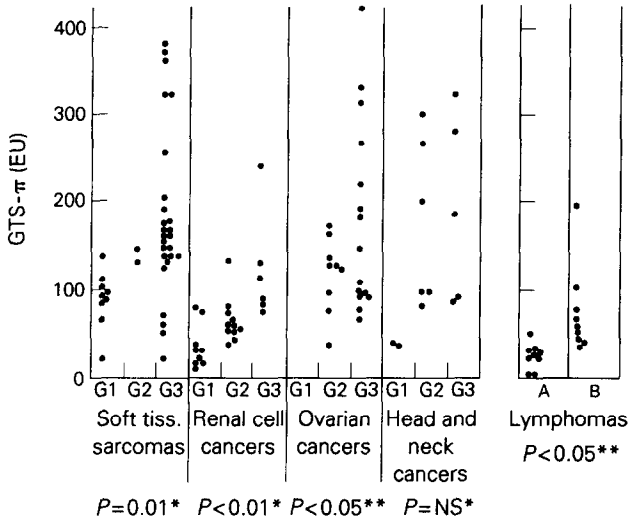

Fig. 3. Relationship between histologic grade and GST-π mRNA EU. *P* values were determined according to the Kruskall–Wallis (*) and Mann-Whitney (**) non-parametric tests. On the right of the figure level of GST-π mRNA is shown in well-differentiated low grade (A) and poorly differentiated NHLs (B).

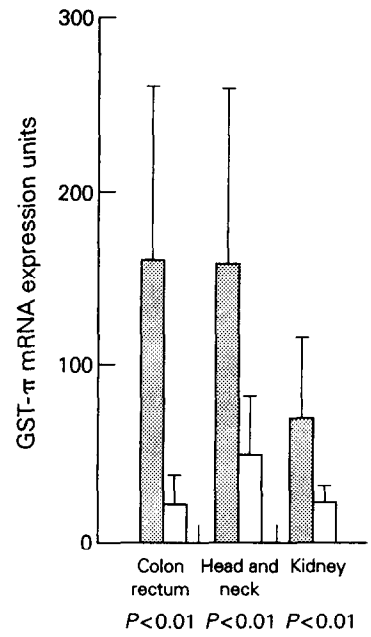

Fig. 4. Mean (S.D.) (bars) GST-π mRNA EU in colorectal, head and neck, and renal cell carcinomas (black columns) and corresponding normal tissues (white columns).

significant correlation between histological grade and GST-π mRNA expression level was observed in head and neck carcinomas (Fig. 3). Such analysis could not be performed in colorectal carcinomas since 27 out of the 29 tumours belonged to a single histological grade group (G2).

Ovarian carcinomas and soft tissue sarcomas were collected from both untreated and chemotherapeutically-treated patients. 12 ovarian carcinomas and 24 soft tissue sarcomas were obtained from untreated patients whereas 12 ovarian carcinomas and 12 soft tissue sarcomas derived from chemotherapeutically-treated patients. No significant difference in GST-π mRNA expression level between untreated and chemotherapeutically treated tumours was evidenced (Fig. 2).

From colorectal, kidney and head and neck carcinoma patients, matched normal and tumoral specimens were available. GST-π mRNA expression level was significantly higher in tumoral specimens than in the normal counterparts ($P < 0.01$) (Fig. 4).

Correlation between GST-π mRNA expression level and tumour replicative activity

Histone H3 mRNA expression level was used as a suitable indicator of the tumoral replicative activity, since this expression

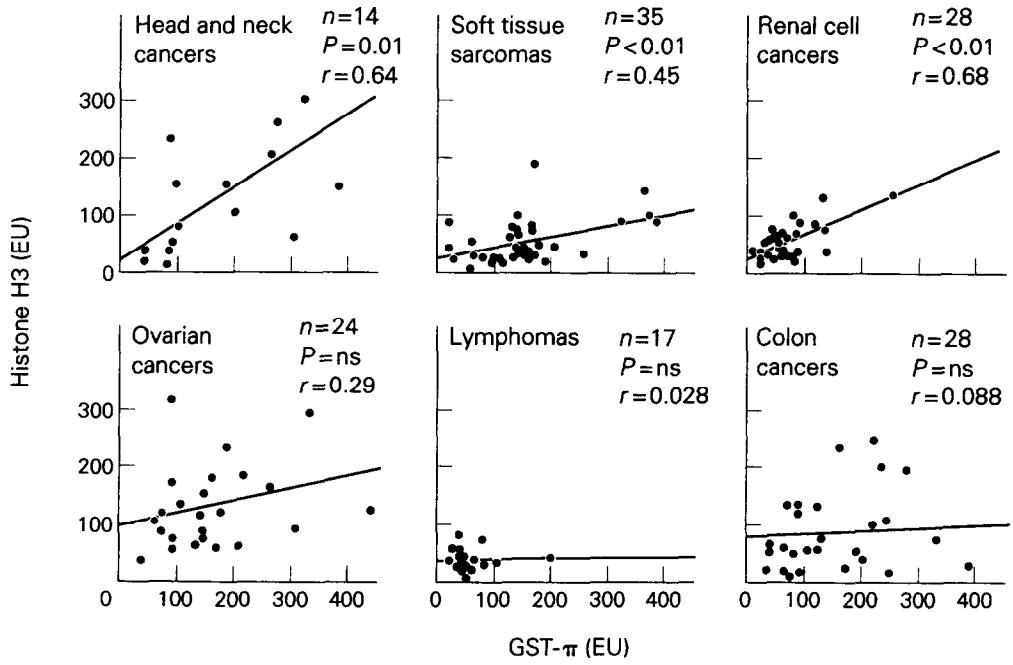

Fig. 5. Scatter diagrams of GST-π mRNA EU in relation to histone H3 mRNA EU. *n*, number of samples; *P*, statistical significance; *r*, coefficient of correlation.

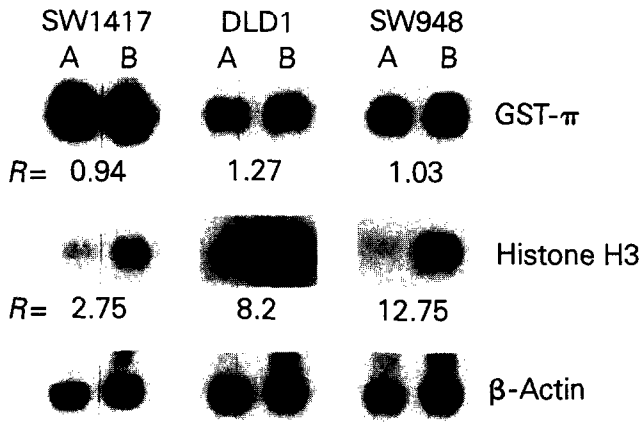

Fig. 6. GST-π and histone H3 mRNA expression in contact-inhibited (A) and exponentially growing (B) colon carcinoma cell cultures. *R*, ratio between mRNA expression level in exponentially growing and contact-inhibited cells.

is restricted to the S-phase of the cell cycle and strictly associated with DNA replication [25]. Histone H3 mRNA expression levels were determined by dot blot and northern blot hybridisation analyses and values were corrected for β-actin hybridisation signal (Fig. 1). Head and neck carcinomas, soft tissue sarcomas and renal cell carcinomas showed a statistically significant direct correlation between GST-π mRNA expression levels and replicative activity ($P < 0.01$) (Fig. 5). On the contrary, such correlation was not statistically significant for colorectal carcinomas, NHL and ovarian cancers.

Correlation between GST-π mRNA expression level and histone H3 mRNA expression level was also investigated in DLD1, SW948 and SW1417 human colon carcinoma cell lines. As shown in Fig. 6, GST-π mRNA was expressed at almost similar levels in exponentially growing and contact-inhibited cell cultures, whereas histone H3 mRNA was expressed at levels considerably higher in exponentially growing than in contact inhibited cells.

MDR1 mRNA expression in untreated and chemotherapeutically treated ovarian carcinomas and soft tissues sarcomas

For ovarian carcinomas and soft tissue sarcomas, tumour samples from untreated and chemotherapeutically treated patients were available. MDR1 and mRNA expression was determined by dot blot hybridisation using the 1.2 kb *Eco*RI fragment derived from plasmid pHuP170#1 as probe (Fig. 1). Moreover, since the pHuP170#1 probe lacks specificity in discriminating between MDR1 and MDR2/3 genes, dot blots were subsequently hybridised with HepG2-16 probe specific for MDR2/3 gene. No hybridisation signal was obtained with the latter probe in any of the tumours tested (data not shown).

Chemotherapeutically treated tumours exhibited a higher frequency of MDR1 mRNA expression than the untreated ones. Moreover, MDR1 mRNA expression levels were significantly higher in pretreated tumours than in those derived from untreated patients ($P < 0.01$ and $P < 0.05$ for ovarian carcinomas and soft tissue sarcomas, respectively) (Fig. 2).

MDR1 mRNA expressing tumours did not exhibit MDR1 gene amplification or structural rearrangements at the Southern blot analysis (data not shown).

DISCUSSION

GST-π mRNA expression level was used as an indicator of GST-π isozyme quantity in both normal and neoplastic specimens. This is mainly because GST-π isozyme cannot be easily distinguished from the other isozymes of the GST family by enzymatic activity determination [8].

On the basis of the mean GST-π mRNA expression level, the tumours analysed can be divided into two groups: high-expressing tumours (ovarian, coltrectal, head and neck cancers and soft tissue sarcomas) and low-expressing tumours (renal cell carcinomas and NHL). However, it is valid only on an average basis since GST-π mRNA expression level both in tumours and normal tissues was scattered over a wide range of values [10]. Matched normal and tumoral specimens were obtained from colorectal, head and neck, and renal cancer patients. For all these tumour types, neoplastic tissues displayed higher GST-π expression levels than those expressed by their corresponding normal tissues (7.3-, 3.5- and 3.0-fold, respectively), supporting the previously described relationship existing between high GST-π mRNA expression level and neoplastic transformation [7–10]. The differential increases suggest that further factors may affect GST-π gene expression [26,27]. Such a possibility is also supported by the finding that renal cell and colorectal carcinomas, derived from tissues with a similar mean basal GST-π mRNA expression level (about 22 EU), displayed highly different, GST-π mRNA expression levels, 63.8 (47.8) and 148.4 (96.4) EU, respectively ($P < 0.01$).

The differentiation grade of the neoplastic cell may be one of the factors affecting GST-π gene expression level, at least in specific tumour types. A significant relationship was, in fact, observed between high histologic grade and high GST-π mRNA expression level in soft tissue sarcomas ($P < 0.01$), renal cell carcinomas ($P = 0.01$) and ovarian cancers ($P < 0.05$). Analogously, well-differentiated low-grade NHLs expressed GST-π mRNA at lower levels than NHLs with more aggressive features ($P<0.05$). On the contrary, no relationship between GST-π mRNA expression levels and histological grade could be observed in head and neck cancers. However, no definitive conclusions for such tumours, can be drawn at this time because of the small number of patients in our study.

Correlation analysis between GST-π mRNA expression levels and replicative cellular activity produced conflicting results. A meaningful relationship ($P< 0.01$) between GST-π mRNA and histone H3 mRNA expression levels was observed in head and neck cancers, soft tissue sarcomas and renal carcinomas, whereas no relationship was evidenced in colorectal carcinomas, NHLs and ovarian cancers. To clarify this issue better, a possible correlation between GST-π and histone H3 mRNA expression level was investigated in an *in vitro* experimental model using contact-inhibited and exponentially growing human colon carcinoma cell lines. Contact-inhibited cells expressed GST-π mRNA expression levels similar to those expressed by the corresponding exponentially growing cell cultures, whereas histone H3 expression level was significantly less expressed. Therefore, GST-π mRNA expression level in neoplastic cells might be more consequent upon the transformed status than cellular replicative activity. Lack of correlation between GST-π mRNA expression level and replicative activity also occurs in normal tissues. In fact, normal kidney and colorectal mucosa, which have very different replicative activities [28], displayed similar GST-π mRNA expression levels.

Previous works have produced conflicting findings about involvement of GST-π activity in drug resistance of human tumours [14, 29, 30]. Our data indicate that GST-π mRNA expression level was not affected by pharmacological treatments, at least in ovarian carcinomas and soft tissue sarcomas. On the

contrary, drug treatments significantly enhanced MDR1 mRNA expression level in these tumours. Therefore, it seems possible to exclude inducibility of GST-π gene expression by pharmacological treatment, at least in the considered tumours studied. It is worthwhile to consider, however, that GST-π uninducibility is not synonymous with a lack of involvement in drug resistance.

In conclusion, neoplastic cells, whether pharmacologically treated or not, seem to require a higher GST-π mRNA expression level than the corresponding normal cells and such a requirement is much more evident in the poorly differentiated neoplastic cells. These findings may suggest that enhancement of GST-π transcriptional activity is an adaptive mechanism set off by the cell to overcome endogenous metabolic disorders, either consequent upon a neoplastic transformation or lack of balance in differentiation-dependent biochemical functions.

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Acknowledgements—This study was supported by a grant from the Italian Association for Cancer Research, Milan, Italy.

We thank Dr M Muramatsu for providing glutathione-S-transferase-π probe, Dr McGuire for MDR1 probe, Dr A.M. Van der Bliek for MDR3 probe and Dr S. Ferrari for histone H3 probe. We also thank Mrs M. Caruso for helping with the English version and Miss P. Pistello for her help with the manuscript.