

GLUTATHIONE TRANSFERASE GST π IN BREAST TUMORS EVALUATED BY THREE TECHNIQUES

RAFAEL MOLINA[†], STEFFI OESTERREICH[§], JIAN-LIANG ZHOU[‡], ATUL K. TANDON[§],
GARY M. CLARK[§], D. CRAIG ALLRED[§], ALAN J. TOWNSEND^{**}, JEFFRY A. MOSCOW^{***},
KENNETH H. COWAN^{***}, WILLIAM L. MCGUIRE[§], SUZANNE A.W. FUQUA^{* §}

[†]Hospital Clinico Provincial, Barcelona, Spain

[§]University of Texas Health Science Center, San Antonio, Texas, U.S.A.

[‡]University of Maryland, Baltimore, Maryland, U.S.A.

^{**}Bowman Gray School of Medicine, Winston-Salem, North Carolina, U.S.A.

^{***}National Cancer Institute, Bethesda, Maryland, U.S.A.

SUMMARY

The glutathione transferases are involved in intracellular detoxification reactions. One of these, GST π , is elevated in some breast cancer cells, particularly cells selected for resistance to anticancer agents. We evaluated GST π expression in 60 human breast tumors by three techniques, immunohistochemistry, Northern hybridization, and Western blot analysis. There was a significant positive correlation between the three methods, with complete concordance seen in 64% of the tumors. There was strong, inverse relationship between GST π expression and steroid receptor status with all of the techniques utilized. In addition, there was a trend toward higher GST π expression in poorly differentiated tumors, but no correlation was found between tumor GST π content and DNA ploidy or %S-phase. GST π expression was also detected in adjacent benign breast tissue as well as infiltrating lymphocytes; this expression may contribute to GST π measurements using either Northern hybridization or Western blot analysis. These results suggest that immunohistochemistry is the method of choice for measuring GST π in breast tumors.

KEYWORDS GST π Breast cancer

INTRODUCTION

Resistance to chemotherapeutic agents is one of the most important problems in cancer treatment with much effort being directed at identifying the mechanisms involved in both intrinsic and acquired drug resistance. The glutathione-S-transferase (GST) enzymes mediate a variety of normal detoxification reactions in cells (Mannervik, 1985), and may represent potential drug resistance mechanisms exploited by tumor cells. The three classes of GST isoenzymes (alpha, mu, and π) are encoded by different genes (Clapper and Tew, 1989) and are associated with different types of drug resistance. For example, increased GST alpha expression is associated with resistance to nitrogen mustards (Lewis *et al.*, 1988) and mechlorethamine (Buller *et al.*, 1987), whereas GST π is elevated in adriamycin-resistant human breast cancer cells (Batist *et al.*, 1986) and human lung tumors (Volm *et al.*, 1992) and carcinogen-induced rat typerplastic liver nodules resistant to a variety of xenobiotics (Cowan *et al.*, 1986). However, the exact role

* Address for correspondence: Suzanne A.W. Fuqua, Department of Medical Oncology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7884.

of the various GST isoenzymes in resistance to specific chemotherapeutic agents remains to be established; it is known that elevated expression of GST π alone in mammalian transfectants does not confer resistance to a variety of drugs and its presence may only be a marker of the drug-resistant phenotype (Moscow *et al.*, 1989a).

GST π is the most prevalent of the GST isoenzymes present in many human tumors with generally higher levels in tumor as compared to matched normal controls (Moscow *et al.*, 1989b; Kodate *et al.*, 1986). In particular, the GST π content in gastric cancers and colon carcinomas is elevated as compared to normal surrounding tissue suggesting that GST π may be useful as a marker of malignant transformation (Kodate *et al.*, 1986). Recently, it has been reported that elevated serum levels of GST π may also be useful for monitoring patients with cancers of the stomach, esophagus, and colon (Tsuchida *et al.*, 1989).

The finding that GST π RNA expression inversely correlated with estrogen and progesterone receptors in primary breast tumors (Moscow *et al.*, 1988a; Gilbert *et al.*, 1993) suggested that measurement of this isoenzyme may be valuable in breast cancer prognosis because it is this group of tumors which traditionally have a poorer outcome and shorter disease-free survival (McGuire, 1978). However, since significant amounts of GST π can be found in many normal tissues (Moscow *et al.*, 1989b), including breast epithelium (Terrier *et al.*, 1990), we questioned whether normal breast tissue GST π expression would contribute to overall GST measurements. We therefore evaluated GST π expression in 60 primary breast cancers by three different techniques; our overall goal was to correlate these results with variables known to be associated with clinical outcome. There was a good correlation between Western blot (WB), Northern hybridization (NH), and immunohistochemical (IHC) techniques for measuring GST π expression. However, benign breast tissues and normal lymphocytes also expressed GST π . Therefore, GST π expression by these normal tissues may contribute to overall GST π measurements. Confirming earlier reports, there was a significant inverse relationship between GST π and steroid receptor status using any of the three detection methods.

MATERIALS AND METHODS

Human breast tumor specimens

Breast tumor biopsies from 60 patients with primary disease were frozen in liquid nitrogen immediately after excision, pulverized, analyzed for steroid receptors (Dressler *et al.*, 1988), and stored in the San Antonio Breast Tumor Data Network at -70°C until required for GST π assessment. Specimens were considered ER-positive if they contained at least 3 fmol of specific binding per milligram of cytosolic protein, and PgR-positive if they contained at least 5 fmol per milligram of cytosolic protein. DNA flow cytometry was performed using 100 mg of each specimen as previously described on an Epics IV flow cytometer (Coulter Electronics, Hialeah, Fla.) (Clark *et al.*, 1989).

Immunohistochemistry

Fifty-five of 60 tumors utilized in this study had sufficient tissues to perform IHC. Briefly, permanent-sections were prepared as previously described (Allred *et al.*, 1990) by rehydrating 50 mg of frozen particulate breast tumor at room temperature in PBS, fixing in 10% formalin for 4 hours, pelleting the particles into a tissue "button" in agar

by centrifugation, infiltrating and embedding the button in paraffin, and cutting sections containing an average of about 500 intact tumor cells. Histological and nuclear grading of tumors were performed using the criteria of Fisher *et al.* (Fisher *et al.*, 1980).

Immunostaining was performed using a standard avidin-biotin-peroxidase complex (ABC) technique (Hsn *et al.*, 1981). Briefly, dewaxed sections were washed in phosphate buffered saline (PBS), and endogenous peroxidase was quenched with 0.1% sodium azide/3% H₂O₂ in PBS for 30 min at room temperature. Sections were washed in PBS and incubated for 30 min in 5% normal goat serum/10% ovalbumin to block non-specific protein biotin. Sections were then incubated at room temperature for 2.5 h with a GST π specific rabbit polyclonal antiserum (Terrier *et al.*, 1990) at a 1/400 dilution in 3% ovalbumin/PBS. After washing in PBS, sections were then incubated with biotinylated swine anti-rabbit secondary antibody (Vector Laboratories, Burlington, GA) at a dilution of 1:200 in PBS for 30 min. Following an additional PBS wash, sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlington, GA), washed again and incubated 5 min in diaminobenzidine/H₂O₂ chromogen substrate. After washing and counter-staining with Harris hematoxylin, samples were dehydrated through graded alcohols and xylene, and mounted with Permount. Cytospins of the Hs578T human breast cancer cell line known to express GST π (Moscow *et al.*, 1988a) were used as positive controls.

Western blot analysis

All of the 60 breast tumors were examined by WB analysis. Approximately 10 mg of tumor powder was exposed to 5% sodium dodecylsulfate (SDS) as previously described (Tandon *et al.*, 1989). Samples were then vortexed, boiled for 5 min, and allowed to cool to room temperature for 15 min. Clear supernatant was collected after centrifugation at 13,000g for 2 min at room temperature. Protein concentration was then determined by the bicinchoninic acid method (Smith *et al.*, 1985).

Tumor protein (100 μ g) was electrophoresed on a 10% polyacrylamide gel under denaturing reducing conditions as described by Laemmli (Laemmli, 1970). Resolved proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 200 mAmp for 16 h at 4°C by the Towbin method (Towbin *et al.*, 1979). After blocking with 5% Carnation milk for 1 h, the blots were incubated with GST π antiserum overnight at 4°C. ¹²⁵I labeled Protein G₁ antibody (200,000 cpm/ml) (Amersham, Arlington Heights, IL) was used for developing the primary antibody and was incubated for 3 h. After washing, the blots were exposed overnight to film at -70°C using intensifying screens. The level of GST π protein in individual tumors was determined by densitometric scanning in a DU-7 spectrophotometer (Beckman, Fullerton, CA) and expressed in densitometric units relative to the densitometric signal from 100 μ g of the Hs578T cell line standard run simultaneously on each gel.

Northern hybridization analysis

Total cellular RNA sufficient for NH was isolated from 57 of the 60 specimens using a Model 340A nucleic acid extractor (Applied Biosystems Inc., Foster City, CA). Quantitation by absorption spectroscopy at 260 nm was confirmed by inspection of an ethidium bromide-stained agarose gel. Twenty μ g of RNA per sample was electrophoresed on a 1% agarose gel containing 0.66 M formaldehyde in 20 mM 3-[N-morpholino]propanesulfonic acid (MOPS) buffer, and transferred to nylon membranes

(Schleicher and Schuell). Hybridization was performed at 50°C overnight in 50% formamide/5X 0.15 M NaCl/15 mM trisodium citrate (SSC)/5X Denhardt's/0.5% SDS/100 µg/ml denatured salmon sperm DNA, and ³²P-labeled GSTπ-1 cDNA (Moscow *et al.*, 1988a) prepared by random primed labeling (Boehringer Mannheim, Indianapolis, IN). After hybridization the membranes were washed with 0.1X SSC/1% SDS at 50°C and autoradiographed. The same filter was hybridized with the pHFb-actin cDNA probe (Gunning *et al.*, 1983) to control for equivalent RNA loading. The intensity of the GSTπ hybridization signal relative to β-actin signal was obtained for each sample by scanning densitometry.

RESULTS

Evaluation of GSTπ expression by three different techniques

Sixty primary breast tumors were chosen for study where sufficient tumor material (>235 mg) was available for analysis. WB analysis was performed first using a rabbit polyclonal anti-GSTπ antibody specific for GSTπ (Terrier *et al.*, 1990). An extract from the Hs578T cell line was also included on each gel as an arbitrary internal reference standard. A single band at approximately 23,000 daltons molecular weight was detected with the antibody (Figure 1, middle panel). GSTπ concentrations by WB were found to vary widely between 0 to 3350 densitometric units/100 µg of sample protein corrected for the signal obtained for 100 µg of control cell line extract.

Sufficient RNA to perform NH analysis was then isolated from 57 of the 60 tumors. A single band at approximately 1.1 kb was detected with the GSTπ cDNA probe (Figure 1, top panel). GSTπ mRNA levels were determined by densitometric scanning and expressed relative to the signal obtained with β-actin (results not shown); mRNA levels also varied widely in the tumors ranging from 0 to 668 densitometric units. There was a good general agreement between these two techniques, but a direct comparison of GSTπ levels required us to define a cut-off for low versus high expression.

Therefore, we undertook to further examine GSTπ protein expression by IHC. IHC results were obtained on 55 of the 60 tumors. Representative staining obtained with the GSTπ specific antibody is shown in the lower panel of Figure 1. Sixty-nine percent of the specimens showed specific cytosolic staining (defined as >5% positive tumor cell staining). Thus for correlative purposes the 69th percentile was used as the cut-off for positive GSTπ expression measured by either NH or WB. The three methods were then compared using chi-square analysis; these results are shown in Table 1. There was a significant positive correlation between the three methods used for GSTπ detection. The closest correlation ($p=0.0001$) was obtained with the two methods (NH and WB) requiring homogenization of the tumor specimens, and whose levels were obtained by densitometric quantitation. There was complete concordance in 64% of the tumors; 54% of the tumors were positive by all of the three methods and 10% were negative for GSTπ expression (Figure 2).

GSTπ expression in benign breast tissues and lymphocytes

The pulverized tumor samples used for tests in this study were initially manually dissected from the fresh surgical specimens. Every effort was made to select "pure" tumor and, therefore, the samples are primarily composed of malignant rather than benign tissue elements. However, twelve of 55 samples (22%) examined histologically

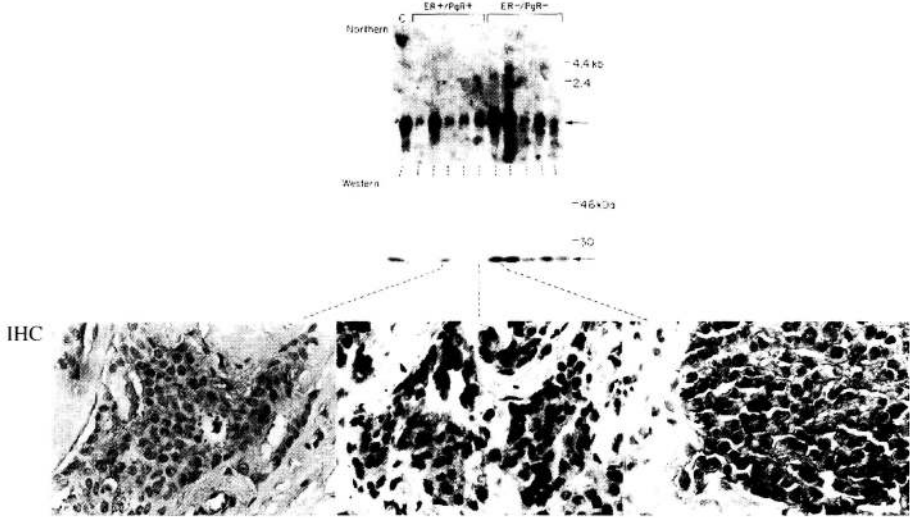


Figure 1. Evaluation of GST π expression by three different techniques. Tumors were simultaneously analyzed by NB (upper panel), WB (middle panel), and IHC (lower panel). Five receptor-positive and five receptor-negative tumors are shown; the positive control cell line (C), Hs578T is also included. Molecular weight standards were run and are given in kilobase pairs (kb) in the NB and kilodaltons (kDa) in the WB.

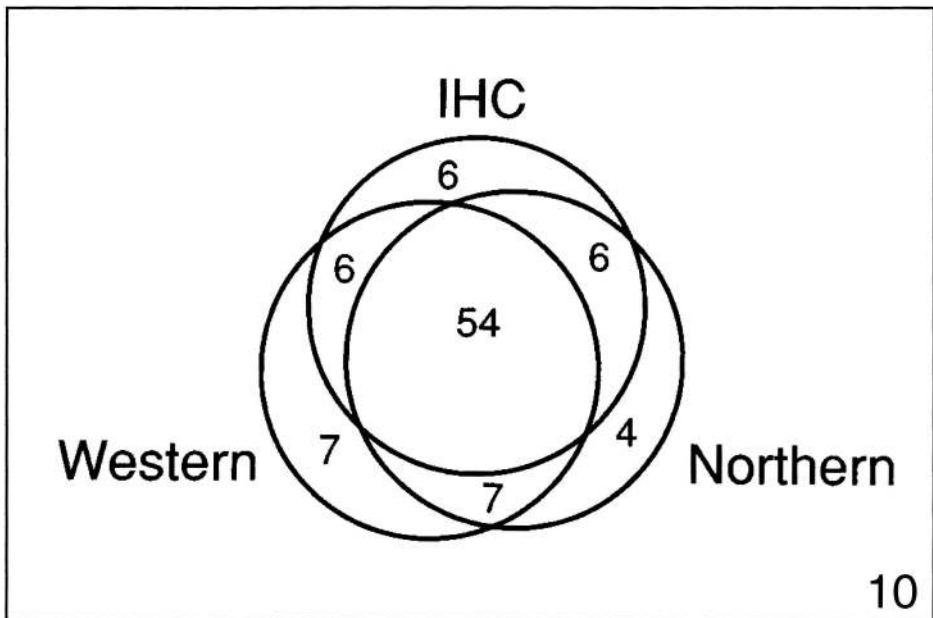


Figure 2. Concordance of GST π expression using the three detection methods. 52 breast tumors were analyzed either by IHC, Western blot, or Northern hybridization. The percent of tumors positive for expression concordantly with the various techniques is shown. 10% of the tumors were negative for GST π as demonstrated.

Table 1. Comparison of NB, WB, and IHC measurements of GST π expression in human breast tumors

	NB		NB		WB	
	Low	High	Low	High	Low	High
Low	11	4	9	6	8	9
WB			IHC		IHC	
High	7	35	8	29	7	31
	p=0.0001		p=0.0075		p=0.0275	

contained some benign breast epithelium (ducts and/or lobules) which, on average, accounted for less than 5% of total sample cellularity. Only about 10% of benign cells within these cases showed a positive IHC signal for GST π (Figure 3) and this signal was generally weak, suggesting that it is unlikely that benign epithelium made a significant contribution to the GST π signal in WB and NH analyses of the same samples. Connective tissue elements (i.e. endothelium, fibroblasts, etc.) within the specimens never showed positive immunostaining (Figure 3, panel C).

Fifty-eight percent of our samples contained tumor infiltrating lymphocytes which, on average, accounted for about 10% of total cellularity in these samples. Most lymphocytes present showed variably intense immunostaining for GST π (Figure 3, panel B), suggesting that lymphocytes could make a significant contribution to the total GST π signal obtained in WB or NH analyses.

Relationships between GST π expression and other prognostic variables of known significance in breast cancer

Associations between GST π and other biological indicators used in breast cancer prognosis and treatment are shown in Table 2. There was a strong, significant inverse relationship between GST π expression and steroid receptor status using all of the three methods with higher GST π expression generally seen in the receptor-negative group of tumors. In addition, there was a trend toward higher GST π expression in poorly differentiated histological grade III and nuclear grade III tumors. No correlation between GST π and ploidy or %S-phase was found.

DISCUSSION

During the last two decades, substantial progress has been made in the development of more effective treatments for cancer. Unfortunately, in the majority of cases, increased response rates have not translated into marked improvements in survival. Resistance to multiple chemotherapeutic agents remains a major obstacle to successful cancer chemotherapy. GST enzymes play an important role in normal cellular defense against toxic

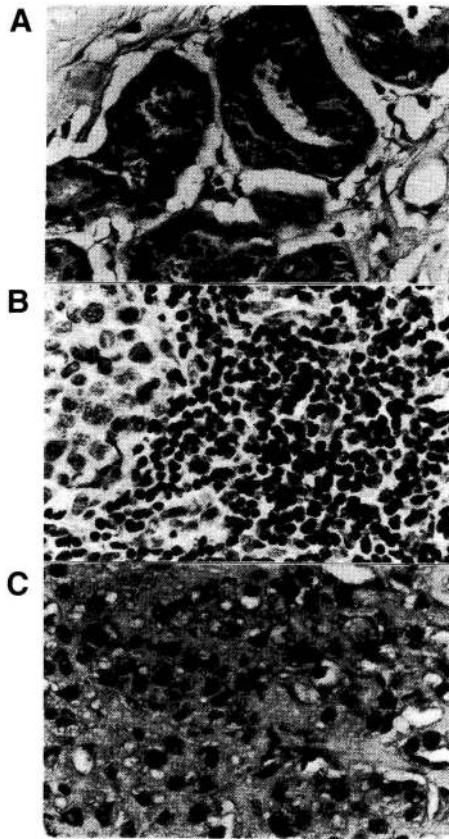


Figure 3. GST π expression in benign breast tissues and lymphocytes. IHC staining of GST π in benign breast epithelium (panel A) and infiltrating lymphocytes (panel B). Breast tumors negative for GST π staining are shown in panels B (arrow) and C.

xenobiotics and carcinogens. These enzymes have also been implicated in the detoxification of many antineoplastic agents (Mannervik, 1985; Clapper *et al.*, 1989; Lewis *et al.*, 1988; Buller *et al.*, 1987; Batist *et al.*, 1986; Evans *et al.*, 1987; Nakagawa *et al.*, 1988), and have been reported to be markers of neoplastic transformation. GST isoenzyme levels, including GST π , are elevated in many human tumors relative to the corresponding normal tissues (Moscow *et al.*, 1989b; Kodate *et al.*, 1986; Tsuchida *et al.*, 1989). A significant increase in GST π activity has been found in tumor as compared to normal adjacent or benign breast lesions (Di Ilio *et al.*, 1985), suggesting that GST π may be involved both in breast cancer development and possibly drug resistance. Therefore, we have begun to measure GST π in breast tumors with the eventual goal being to evaluate its expression as a marker during breast cancer progression.

Due to the availability of both specific GST π antibodies and cDNA probes, we analyzed GST π expression at both the protein and mRNA levels. Two of the methods chosen, WB and NH, share the advantage of being semiquantitative, but require homogenization of the tumor. These methods are then subject to experimental error due

Table 2. Relationship of GST π expression and other prognostic variables in breast cancer

PROGNOSTIC VARIABLE	% TUMORS WITH HIGH GST π		
	NB	WB	IHC
ER- PGR-	87 *** ***	83 ***	86
ER+ PgR+	46	62	50
Histological grade I-II	48 *** *	70	61
III	86	76	86
Nuclear grade I-II	50	70	58
III	73 *	73	80
Diploidy	64	70	68
Aneuploidy	82	83	70
S-phase $\leq 6.7\%$	58	67	50
$>6.7\%$	75	67	71

*** significant, $p < 0.05$ * $0.05 < p < 0.09$

to the dilution of tumor extracts with surrounding normal breast, vascular elements, or inflammatory cells which are often present in breast tumor specimens. However, in spite of these limitations we wanted to use semiquantitative methods so as to fully evaluate associations between GST π levels and known biological parameters commonly delineated in breast cancer.

Confirming earlier reports (Tsuchida *et al.*, 1989, Howie *et al.*, 1989), we observed an inverse relationship between GST π expression and the expression of estrogen and progesterone receptors using all three of the detection methods. Similar results have been reported with the epidermal growth factor receptor where elevated expression is also seen in the receptor-negative group of patients (Cappelletti *et al.*, 1988; Sainsbury *et al.*, 1985; Delarue *et al.*, 1988). Recently, it has been suggested that the estrogen receptor may exert a constitutive repressor function on estrogen-responsive genes in the absence of hormone (Tzuckerman *et al.*, 1990). It is an intriguing hypothesis that the observed inverse relationship between GST π expression and the presence of the estrogen receptor may be related to this repressor activity. Although, the recent analysis of the promoter elements and the posttranscriptional fate of GST π (Morrow *et al.*, 1992) has shown that the differential expression in ER+ versus ER- is governed by posttranscriptional processes.

There was also a trend towards higher GST π expression in poorly differentiated tumors. Several studies have detected a relationship between the receptor-negative phenotype and the degree of cellular dedifferentiation (Fisher *et al.*, 1981b), thus the interrelationships we report here are a further demonstration of the basic biological differences between receptor-positive and receptor-negative breast cancers. Future studies should be directed at identifying common regulatory factors that may underlie these associations.

The value of estrogen receptors in predicting the endocrine response of breast cancer has been appreciated for some time (Osborne *et al.*, 1980). Steroid receptors not only are valuable for predicting response to hormonal manipulation, but also the time course of the disease (Osborne *et al.*, 1980; Benner *et al.*, 1988). Therefore, combining receptor status with other parameters such as GST π or histopathology may provide very valuable treatment guides. However, a larger study of breast cancer specimens with adequate clinical follow-up will be required to address these issues. Additionally, the clinically important question whether GST π is directly involved in chemotherapeutic resistance remains unanswered to date.

IHC assessment of GST π expression in breast cancer biopsies demonstrated specific cytosolic staining in two types of non-malignant cells; both normal mammary epithelium and lymphocytes. These benign cells may be present in heterogenous breast tumor specimens. Twenty-two percent of the cases in our series contained benign epithelium which, on average, accounted for less than 5% of the cells within the sample. Furthermore, only about 10% of benign cells showed positive immunostaining for GST π . Therefore normal breast epithelium does not appear to make a major contribution to overall GST π measurements by NH or WB. In contrast, GST π positive lymphocytes were present in the majority of samples, which has been reported previously (Del Boccio *et al.*, 1986). Thus, lymphocyte infiltration may make a contribution to the overall measurement of GST π using methods that are unable to differentiate the cellular source of GST π in the tumor. This may be one reason why other studies have not detected correlations between GST π and some prognostic factors (Shea *et al.*, 1990). We feel that

IHC should probably always be included in an analysis of GST π content in breast cancer. By itself, IHC provides significant information regarding the relationship of GST π to other biological characteristics of breast cancer. In addition, IHLc can discriminate the cell source of GST π expression, enabling accurate interpretation of more quantitative WB or NH analyses.

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