Int. J. Cancer: **109,** 717–720 (2004)
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Publication of the International Union Against Cancer

Nup88 mRNA OVEREXPRESSION IS ASSOCIATED WITH HIGH AGGRESSIVENESS OF BREAST CANCER

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The nuclear pore complex protein Nup88 is overexpressed in tumor cells. Immunohistochemical studies have shown that this overexpression is linked to higher aggressiveness of colorectal carcinoma and to enhanced metastatic potential of melanoma cells. However, the antibodies so far developed against Nup88 have the drawback of recognizing a number of other, up to now unspecified antigens besides Nup88. For this reason, we devised the present study on Nup88 expression at the mRNA level. RNA was extracted from fresh tumor tissue corresponding to 122 breast cancer patients. *Nup88* mRNA expression was measured by means of differential RT-PCR, standardizing against a constitutive internal control gene (*β-actin*). The results were dichotomized into "high" and "low" expression levels, using the median value as cut-off. High *Nup88* mRNA expression levels correlated significantly with ductal and tubular histology ($p = 0.012$), histologic and nuclear grade 3 of tumors ($p < 0.001$), absence of hormone receptor expression ($p < 0.001$), expression of the *c-erb-B2* oncogene ($p < 0.001$), expression of mutant p53 protein ($p < 0.001$), high proliferation (defined by Ki67 labeling index >20%, $p < 0.001$), DNA aneuploidy ($p < 0.001$) as well as the most important ominous clinical prognostic factor, axillary node invasion ($p < 0.001$). We also found an inverse correlation ($p < 0.001$) with expression of the *H-MAM* (mammaglobin) gene, a marker of low biologic and clinical aggressiveness of breast cancer. All of these factors, without exception, define a highly aggressive tumor phenotype. These findings appear to be specific to Nup88 and not to nuclear pore proteins in general. Indeed, analysis of Nup107 (which is a limiting component of the nuclear pore complex) under the same conditions in the same tumors did not yield comparable results.
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Key words: *breast cancer;* Nup88; *aggressiveness*

The nuclear pore complex protein Nup88, identified concomitantly by Fornerod *et al.*[1] and Bastos *et al.,*[2] is the antigen recognized in human tumors by the monoclonal antibody (MAb) C6,[3] which also cross-reacts with the yeast *Candida albicans,* as described by us in a previous report.[4] In the course of the investigation leading to the recognition that Nup88 is overexpressed in tumor cells, we also developed a polyclonal antibody against Nup88, which has been used in further studies. In them, it has been shown that Nup88 is overexpressed in virtually all tumor tissues[5] and, furthermore, that this overexpression is linked to a higher aggressiveness of colorectal carcinoma[6] and to enhanced metastatic potential of melanoma cells.[7] However, both our C6 MAb and the polyclonal anti-Nup88 antiserum have the drawback of cross-reacting with several other epitopes, as shown in the corresponding Western blots. This means that we do not exactly know how much of the observed reaction is attributable to Nup88 or, perhaps, to the other cross-reacting and up to now unidentified epitopes. With this in mind, we designed the present study. In it, we analyzed Nup88 mRNA expression by differential RT-PCR in a series of human breast cancers, quantifying it against the expression of a constitutive internal control gene. Although mRNA expression does not necessarily reflect the ultimate translation of a given gene, it is nevertheless 100% specific for it and, thus, eliminates the doubts raised by the lack of specificity inherent in the use of immunohistochemistry. As a final step, we correlated the expression level of Nup88 in each tumor with all its other known biologic and clinical features, to explore the possible biologic significance of the former in human breast cancer.

MATERIAL AND METHODS

Fresh tumor tissue from 122 breast cancer patients operated upon at Fundación Tejerina (Madrid, Spain) was obtained at the time of surgery and immediately snap-frozen or immersed in RNAlater (Qiagen, Hilden Germany). Normal tissue from the same breast containing the tumor was obtained and conserved as described in 17 instances.

The mRNA concentrations of the 139 samples ranged 11.2–238.0 ng/μl.

The histologies of the tumors were as follows: ductal infiltrating, 99; lobular infiltrating, 19; tubular, 4. Of the 122 patients, 14 had received induction chemotherapy prior to surgery, the rest being untreated at the time of operation.

In addition to measuring Nup88 expression, the following parameters were determined in all tumors: DNA ploidy, by means of flow cytometry; hormone receptor (estrogen, progesterone), Ki67, c-erb-B2 and p53 expression, by means of immunohistochemistry (Ki67-labeling index expressed as percentage of specifically staining tumor nuclei; all other parameters were considered positive when >10% of tumor cells showed specific staining); and *h-MAM* (mammaglobin) expression, by means of differential RT-PCR, taking also the median expression value as the cut-off for comparison. All procedures were exactly the same as described in other reports by our group, and the reader is referred to them for details.[8–10] Finally, conventional features of the tumors, *e.g.,* histologic and nuclear grade or axillary lymph node invasion, were also included in the statistical analysis.

RT-PCR

RNA was extracted from tumor specimens using the RNeasy commercial kit (Qiagen), according to the manufacturer's instructions. Total RNA content was immediately quantified in a spectrophotometer (SmartSpec 3000; Bio-Rad, Hercules, CA) after 1:50 dilution in RNAse-free water and the RNA then frozen at −80°C until further use. RT-PCR was carried out in an iCycler thermal cycler (Bio-Rad), using a commercial 1-step RT-PCR kit

Grant sponsor: Fondo de Investigación Sanitaria; Grant number: FIS PI020432.

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Received 8 August 2003; Revised 5 November 2003; Accepted 7 November 2003

DOI 10.1002/ijc.20034

(Qiagen). The mRNA concentration corresponding to each sample was diluted to 10 ng/μl, and 1 μl was used for each reaction. The number of extension cycles of the PCR was adjusted so that it was within the ascending slope of the saturation curve, which was calculated for both tumor and normal tissue, which yielded virtually identical results. The primer concentration of the target and the internal control gene (*β-actin* in this case) was accordingly adjusted so that the final band densities were similar and both reactions were within the ascending slope of the respective saturation curves using the same number of extension cycles (Fig. 1). This allowed for the reactions to be carried out in a single tube. A second tube with the same components, save for the reverse transcriptase, was run in parallel with each reaction, to exclude amplification of a pseudogene from contaminating DNA. The concentrations of the different components, the primers used and the conditions of the RT-PCR are shown in Table I.

Quantification

To quantify the expression levels of the target gene *(Nup88),* 1.5% agarose gels (with 2 μl ethidium bromide/150 ml gel) were run, loading each lane with 10 μl RT-PCR product, for 2 hr at 100 V. They were then analyzed in a UV-transilluminator mini-darkroom (UVP, Upland, CA), by means of Labworks analysis software (UVP). The band density corresponding to the target gene divided by that of the internal control gene gives a figure in arbitrary units, which reflects the relative level of expression of the former.

Statistics

For comparative purposes with all other qualitative variables, *Nup88/β-actin* band density quotients, expressed in arbitrary units, were divided into high and low groups, using the median value as cut-off. Afterward, using this cut-off, *Nup88* expression was correlated with all other biologic and clinical variables in a univariate model (Table II). Nup107, another nucleoporin which is a limiting component of the nuclear pore complex, was also studied in an analogous way in the same tumors, under the same conditions, to determine if the results were attributable to Nup88 in particular or to nucleoporins in general (Table III).

Statistical analysis was performed using the STATA statistical package (Stata, College Station, TX).

TABLE I – PRIMERS AND THERMAL CYCLER CONDITIONS USED (N = 35 CYCLES)

Gene		Primer sequence (5′–3′)	Product (bp)
β-*actin*	ACTB1	GGAAATCGTGCGTGACATTA	378
	ACTB2	GGAGCAATGATCTTGATCTTC	378
Nup88	Nup88a	TAAAGGAAGGGCGTATACCG	276
	Nup88b	AAGCAGAGTACAGCACACGC	276

Step	Temperature	Time (min)
Reverse transcription	50°C	30
Initial PCR activation step	95°C	15
PCR amplification		
Denaturation	94°C	0.5
Annealing	55°C	0.5
Extension	72°C	1
Final extension	72°C	10

RESULTS

Inverse transcription and amplification was successful for all 122 tumors in the case of *Nup88* and for 114 tumors in the case of *Nup107,* which may be attributable to lacking mRNA (or mRNA degradation) since *Nup107* expression was analyzed after completion of the *Nup88* study. A *Nup88* mRNA expression level above the median was significantly more frequent (p = 0.012) in tumors with ductal and tubular histology than in lobular invasive carcinomas. Moreover, the cited level of *Nup88* expression correlated in

TABLE II – CORRELATION OF Nup88 mRNA EXPRESSION IN BREAST CANCER WITH ALL OTHER CLINICAL AND BIOLOGIC VARIABLES OF THE TUMORS (N = 122)

Variable	Number	Nup88 level		
		Median	P25–P75	p
Histology				
Ductal + tubular	103	2.07	0.72–3.58	0.012
Lobular	19	1.05	0.43–1.40	
Histologic grade				
1 + 2	82	1.07	0.44–2.08	
3	40	3.72	2.61–5.63	<0.001
Nuclear grade				
1 + 2	73	1.17	0.41–2.11	
3	49	2.91	1.53–4.97	<0.001
Positive nodes				
No	67	0.91	0.37–2.46	
Yes	52	2.49	1.45–4.51	<0.001
Aneuploidy				
Yes	64	2.67	1.28–4.55	<0.001
No	58	0.91	0.35–2.08	
Estrogen receptor				
Negative	33	3.58	1.92–5.46	<0.001
Positive	88	1.26	0.46–2.34	
Progesterone receptor				
Negative	61	2.64	1.19–4.73	<0.001
Positive	61	1.02	0.38–2.31	
c-erbB2				
Negative	97	1.26	0.54–2.42	
Positive	25	5.04	3.06–6.13	<0.001
p53				
Negative	101	1.33	0.55–2.65	
Positive	21	5.04	2.11–6.50	<0.001
Ki67				
≤20%	76	1.20	0.40–2.36	
>20%	46	2.82	1.28–4.63	<0.001
H-MAM				
≤Median	57	2.60	1.24–4.38	<0.001
>Median	65	0.97	0.44–2.37	

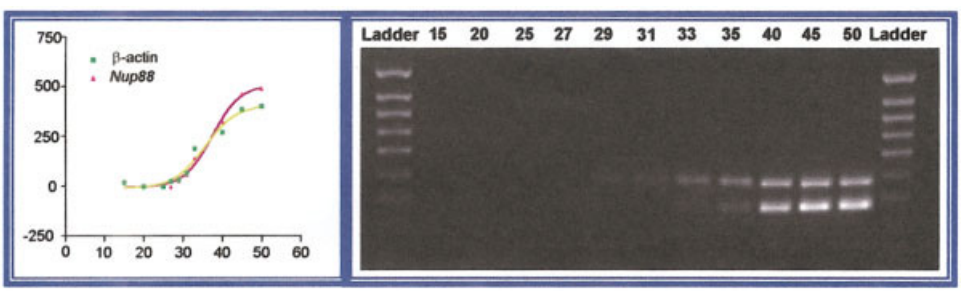

FIGURE 1 – Saturation curves for the study and control genes. *(Curve) X* axis, number of extension cycles. *(Gel)* Lanes between ladders correspond to different extension cycles tested.

TABLE III – CORRELATION OF *Nup107* mRNA EXPRESSION IN BREAST CANCER WITH ALL OTHER CLINICAL AND BIOLOGIC VARIABLES OF THE TUMORS ($N = 114$)

Variable	nup107 level			
	Number	Median	P25–P75	*p*
Histology				
Ductal + tubular	96	1.29	0.93–1.73	0.14
Lobular	18	1.14	0.92–1.43	
Histologic grade				
1 + 2	52	1.29	0.88–1.71	0.511
3	38	1.29	1.04–1.85	
Nuclear grade				
1 + 2	44	1.29	0.96–1.71	0.924
3	47	1.29	0.92–1.80	
Positive nodes				
No	63	1.21	0.90–1.67	0.218
Yes	48	1.29	1.13–1.62	
Aneuploidy				
Yes	60	1.37	1.14–1.88	0.002
No	54	1.14	0.87–1.43	
Estrogen receptor				
Negative	33	1.34	1.07–1.86	0.282
Positive	80	1.25	0.92–1.61	
Progesterone receptor				
Negative	59	1.3	0.95–1.82	0.352
Positive	55	1.25	0.93–1.52	
c-erbB2				
Negative	91	1.26	0.92–1.60	
Positive	23	1.52	1.21–1.93	0.043
p53				
Negative	94	1.28	0.93–1.63	0.991
Positive	20	1.26	0.97–1.80	
Ki67				
≤20%	71	1.28	0.95–1.66	0.921
>20%	43	1.28	0.92–1.64	
h-MAM				
≤median	59	1.3	1.06–1.82	0.085
>median	55	1.21	0.89–1.52	

a highly significant ($p < 0.001$) way with histologic and nuclear grade 3 of tumors, absence of hormone receptor expression, expression of the *c-erb-B2* oncogene and mutant p53 protein, high proliferation (Ki67 labeling index >20%), DNA aneuploidy as well as the most important ominous clinical prognostic factor, axillary node invasion. It also correlated inversely with expression of the *H-MAM* (mammaglobin) gene, a marker of low biologic and clinical aggressiveness of breast cancer.[9] All of these factors, without exception, define a highly aggressive tumor phenotype.

Possible contamination from mRNA coming from the normal cell component present in every tumor does not alter these results since the expression value in all normal tissues studied was always below the median value used as a cut-off for overexpression.

Nup107 expression, however, correlated just significantly with *Nup88* expression ($r = 0.18$, $p = 0.05$) as well as with aneuploidy ($p = 0.02$) and *c-erb-B2* overexpression ($p = 0.042$), but none of the other clinical and biologic parameters correlated in a highly significant way with *Nup88* expression. Taking the previously treated tumors out of the series did not alter the results. The results are summarized in Tables II and III.

DISCUSSION

Our results indicate that *Nup88* overexpression confers, or is closely associated to, higher aggressiveness of breast cancer. This would confirm the previous results obtained by immunohistochemistry in colorectal cancer and melanoma[6,7] and appears not to be a chance finding attributable to overexpression of nucleoporins in general, associated with the higher turnover characteristic of actively dividing tumor cells. Indeed, when studying *Nup107* expression in the same cells, we find a similarity with *Nup88* expression only related to those parameters involved in cell proliferation (*i.e.,*

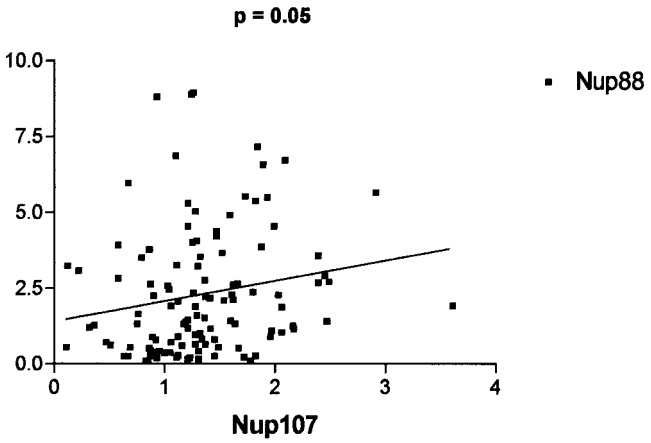

FIGURE 2 – Correlation between expression of Nup88 and Nup107. Linear regression curve ($n = 114$).

ploidy and *c-erb-B2* overexpression), as could be expected since all nucleoporins are overexpressed to some degree during cell division. However, when we look at all other clinical and biologic markers definitive of a highly malignant phenotype in breast cancer (Tables II, III), which were uniformly associated in a highly significant way with *Nup88* overexpression in our breast cancer series, there is no correlation with *Nup107* overexpression. It is, moreover, somewhat surprising that the relationship between the expression of either nucleoporin is, although just significant ($p = 0.05$), relatively weak ($r = 0.18$) (Fig. 2), taking into account that Nup107 expression is considered essential for nuclear pore complex assembly and a surrogate for expression of the remaining components of the nuclear pore complex.[11] Thus, *Nup88* overexpression appears to have a more specific relationship with tumor aggressiveness. What precise role Nup88 plays in this context, however, remains unknown. We can only indirectly speculate from what we know about another nucleoporin, Nup214/CAN, with which Nup88 is closely associated. Indeed, Nup88 forms part of the CAN/Nup complex, which in its turn is located in the cytoplasmic filaments of the nuclear pore complex, and is no longer detectable when the latter is deleted. Furthermore, when the CAN/Nup complex is experimentally deleted, the resulting tumor phenotype shows defective protein transport through the nuclear membrane (in both directions) as well as cell cycle arrest in G_2.[12] Nup214 overexpression, however, is associated with cell cycle arrest in G_0, mRNA accumulation in the nucleus and apoptosis.[13] It therefore appears that at least Nup214 plays some fundamental role related to the correct progression of the cell cycle and that Nup88, being closely linked to Nup214, may also be involved in this process. That indeed both Nup214 and Nup88 may play a role in human cancer is exemplified by the original research on leukemia fusion proteins by Fornerod *et al.,*[1] which ultimately led to the cloning by them of *Nup88.* Indeed, it was shown that the CAN/Nup214 complex is fused to the SET protein, which plays a key role in leukemogenesis. Transport through the nuclear pore, although still little explored in connection with cancer, must necessarily be a crucial event in the development and maintenance of the tumor phenotype. To begin with, all mRNA must pass through it for translation and protein synthesis, and many of the synthesized proteins pass again into the nucleus to interact directly with the DNA as gene regulators, DNA-repair proteins, etc. Both events are especially exacerbated in cancer. As an example, ferritin is present not only in the cytoplasm but also in the nucleus, where it may be involved both in the protection of DNA and, paradoxically, in the exacerbation of iron-induced oxidative damage to DNA.[14] From what we know of other mechanisms of active, energy-consuming membrane transport, this tends to be highly specific, mediated by recognition proteins on both sides of the barrier. Thus, Nup88

might well be involved in such a "gatekeeper" function, and the recognized protein(s) may have a role similar to the one played by ferritin (or another role entirely), ultimately leading to enhancement of the malignant phenotype of breast cancer.

In conclusion, the nuclear pore complex protein Nup88 is expressed in breast cancer. Overexpression is significantly associated to biologic and clinical features of the tumor defining a highly aggressive phenotype, and this appears to be specifically related to Nup88 and not to nucleoporins in general.

ACKNOWLEDGEMENTS

We thank Ms. Beatriz Santamaría for optimal technical assistance.

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