doi:10.4149/neo_2012_029

Immunohistochemical analysis of gastrin-releasing peptide receptor (GRPR) and possible regulation by estrogen receptor βcx in human prostate carcinoma

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Received July 29, 2011 / Accepted October 26, 2011

Gastrin-releasing peptide (GRP) belongs to the family of bombesin-like peptides. GRP was demonstrated to stimulate the proliferation and invasiveness of androgen-independent prostate carcinoma. GRP mediates its action through the membrane-bound receptor, GRP receptor (GRPR), which is characterized by a high-affinity binding for both GRP and bombesin. In human prostate cancer tissue, GRPR mRNA was reported to be detectable in more than 90% but its immunolocalizaition has not been reported. Therefore, in this study we immunolocalized GRPR in 51 human prostate cancer cases and correlated the findings with several clinicopathological parameters in order to beter understand the function and regulation of GRPR in human prostate cancer. GRPR was immnolocalized in carcinoma cells and their values were significantly associated with Gleason score and immunoreactivity of estrogen receptor βcx (ERβcx) that is one of splicing variants of ligand dependent transcription factor, ERβ, and considered to be prognostic factor of prostate cancer patients. The amounts of GRPR and ERβcx mRNA in three prostate cancer cell lines PC-3, DU-145 and LNCaP evaluated by quantitative RT-PCR (qPCR) analysis were also significantly correlated. In addition, we established stable transformants of prostate carcinoma cell line PC-3 introduced with ERβcx, and confirmed that GRPR mRNA was induced in ERβcx over-expressing PC-3 cells by qPCR analysis. These results also suggest that ERβcx contributes to prostate cancer development possibly through mediating GRPR expression in carcinoma cells.

Key words: ERβcx, GRPR, prostate cancer

Gastrin-releasing peptide (GRP) belongs to the family of bombesin-like peptides that includes the amphibian peptide bombesin as well as the mammalian counter-parts GRP and neuromedin B [1]. Several previous studies demonstrated that growth stimulation of bombesin-like peptides played some roles in the process of human carcinogenesis [2-7]. In human prostate cancer, GRP was also reported to increase the proliferation and invasiveness of androgen-independent prostate cancer [8-11]. In addition, the secretion of GRP by neuroendocrine cells has been also proposed to cause prostate cancer progression, development of androgen independence, and poor prognosis [12]. GRP mediates its action through membrane-bound receptors. These receptors correspond to one of the subtypes of the bombesin-like peptide receptors, namely the GRP receptor (GRPR), which is characterized by a high-affinity binding for GRP and bombesin. GRPR also belongs to the members of the large superfamily of G-protein-coupled receptors with seven transmembrane domains. GRPR have been also detected in various types of tumor cell lines including prostate cancer cells [13-16]. In human prostate cancer tissue, GRPR mRNA was also reported to be detected in more than 90% of the cases examined, and to be involved in prostate cancer development by two different investigators [17, 18]. However, GRPR immunolocalizatoin has not been studied in normal human prostate.

In order to further understand the mechanism of functions and regulation of GRPR in human prostate cancer, we immunolocalized GRPR and correlated the findings with clinicopathological parameters including ages, proste specific antigen (PSA) level, Gleason score, stage, lymph node status,

neuroendocrine differentiation (NED) status, Ki67 labeling index (LI), androgen receptor (AR) LI, estrogen receptor β (ERβ) LI, and ERβcx LI. ERβcx is the one of well-studied isoform of ERβ which is estrogen dependent transcription factor, and reported to be expressed abundantly in high-grade prostate carcinoma than low grade one [19,20]. In addition, clinical outcome of patients with high ERβcx was worse than those with lower ERβcx in human prostate carcinoma [20]. Therefore, ERβcx is currently considered as one of the important prognostic factor in the patients with prostate carcinoma. In this study, we compared the status of GRPR with several clinicopathological parameters and demonstrate statistically significant correlation of GRPR status with ERβcx immunoreactivity. Moreover, we established stable transformants of well-documented prostate cancer cell line PC-3 cells with introducing ERβcx vectors to analyze the biological functions of ERβcx. We then performed quantitative RT-PCR (qPCR) analysis in order to confirm whether GRPR was induced in ERβcx over-expressing PC-3 cells.

Materials and Methods

Prostate Carcinoma Cases. 51 archival specimens of prostate carcinoma retrieved from patients who had under-went prostatectomy at Department of Urology, Tohoku University Hospital (Sendai, Japan). The mean age of the patients was 65.2 years (range: 47-77). All patients examined in this study did not receive radiation, chemotherapy, or hormone therapy prior to surgery. Clinical data, including patients' age, serum prostate specific antigen (PSA) concentration, clinical stages according to the International Union Against Cancer TMN classification (1987), lymph node status, and Gleason score were all retrieved from reviewing the charts of the patients. The histlogical grade of each tumor was evaluated by two of the authors (Y.N. and T.S.). All the specimens had been fixed with 10% formalin and embedded in paraffin wax at the Department of Pathology, Tohoku University Hospital. The Ethic's Committee at Tohoku University School of Medicine approved the research protocol for this study (2003-146).

Immunohistochemistry. A Histofine Kit (Nichirei, Tokyo, Japan) that employs the streptavidine-biotin amplification method was used in this study for immunostaining. Antigen retrieval was performed by heating the slides in an autoclave at 120°C for 5min in citric acid buffer (2mM citric acid and 9mM trisodium citrate dehydrate, pH 6.0). Mouse monoclonal antibodies against Ki-67, AR, ERβ and ERβcx were purchased from DAKO Corporation (Glostrup, Denmark), GeneTex(SanAntonio, TX) and Acris Antibodies GmbH (Hiddenhausen, Germany) respectively. Rabbit polyclonal antibody against GRPR and chromogranin A (CgA) was purchased from GeneTex and DAKO Corporation respectively. The dilutions of primary antibodies were as follows: Ki-67, 1/50; AR, 1/100; ERβ, 1/1500; ERβcx, 1/500; GRPR, 1/250 and CgA, 1/2000. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine solution (1mM, in 50mM Tris-HCl buffer (pH7.6) and 0.006% H_2O_2), and counterstained with hematoxylin. As a negative control, normal rabbit or mouse IgG was used instead of the primary antibodies, and no specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity. Ki-67, AR, ERβ and ERβcx immnoreactivity was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. GRPR immunoreactivity was tentatively classified into the following three groups: ++, >=50% positive carcinoma cells; +, 10% to 49% positive cells; and -, 0% to 9% negative cells. Neuroendocrine differentiation of prostate carcinoma cases was evaluated with immunohistochemistry of CgA. CgA immunoreactivity detected in the cytoplasm, and evaluated using a semi-quantitative approach according to the results of previous report [21]. Briefly, carcinoma cases were classified as NED-negative when no CgA-positive cells were detected (scored 0), or when positively stained cells were found to be scattered throughout the tumor without any cluster formation (scored 1+) with cluster being defined as the at least three stained cells situated so closely as to touch cell surface of each other. We defined NED-positive when at least one CgA-positive cluster (scored 2+) or when a few to many clusters (scored 3+) could be identified.

Cell lines and plasmids. Human prostate cancer cell line PC-3, DU-145 and LNCaP were provided from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University(Sendai, Japan). The cell lines were maintained in RPMI-1640 (Sigma-Aldrich, St.Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS). In this study, we used estrogen-responsive reporter plasmids pERE-Luc, containing Xenopus vitellogenin A2 estrogen-responsive element (ERE) [22]. The pRL-TK vectors were purchased from Promega (Madison, WI). Full length of cDNA for ERβ and ERβcx were constructed in pRc/CMV expression plasmid (Invitrogen Corporation, Carlsbad, CA) [23].

Establishment of PC-3 cells associated with ERβcx-overexpression. In order to establish stable transformant expressing high levels of ERβcx protein in PC-3, ERβcx cDNA cloned into pRc/CMV was transfected using *Trans*IT-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI) was used following the supplier's instruction. A control cell line was established by transfecting an empty expression plasmid. The clones expressing high levels of ERβcx mRNA was selected by G-418 (Sigma-Aldrich) treatment. Transfected PC-3 was cultured for 2 weeks with G-418, and clones acquired resistance against G-418 was subsequently isolated. More than 10 stable transformant clones were isolated and the levels of mRNA and protein expression of ERβcx were subsequently confirmed by both qPCR and western blot analysis. Two of the clones, tentatively termed Mock-#1 and Mock-#2, ERβcx-#7 and ERβcx-#8 were used for further analysis, in this study.

Luciferase assay. The luciferase assay was performed according to a previous report with some modification [24]. Briefly, pERE-Luc, pRL-TK and pRc/CMV-ERβ plasmids were used to analyze dominant negative effects of ERβcx expressed in stable transformant. Cells were seeded at 1×10^5 cells/well in 24-well culture plates in RPMI 1640 supplemented with 10% FBS and allowed to adhere for 24hr. Cultures were subsequentry washed twice with PBS. Cells were then cultured for 24hr in phenol-red-free RPMI1640 without FBS for serum deprivation. Transient transfection was carried out using *Trans*IT-LT1 Transfection Reagents (Mirus Bio Corporation), and the luciferase activity of lysates were measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN(AB-2200) (Atto Co., Tokyo, Japan) after incubation with 100nM 17β-estradiol(Sigma-Aldrich) for 24hr. The cells were also treated with the same volume of ethanol (final dilution-0.05%) for 24hr as controls. The transfection efficiency was normalized against Renilla luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.

qPCR analysis. Total RNA was extracted from the cells using TRIzol regents(Invitrogen Corporation), and cDNA was synthesized using QuantiTect reverse transcription kit (QIAGEN GmbH, Hilden, Germany). Real-time PCR was carried out using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The PCR primer sequences of ERβcx, GRPR, and ribosomal protein L13A (RPL13A) used in this study are as follows: ERβcx [AB006589; forward 5'-GATCTTGTTCTGGACAGGGAT-3' and reverse 5'-AGGCCTTTTCTGCCCTC-3'], GRPR [NM_005314; forward 5'-CTGATCCAGAGTGCTTACAA-3' and reverse 5'-CGGTACAGGTAGATGACATGA-3'], RLP13A [NM_012423; forward 5'-CCTGGAGGAGAAGAGGAAAGAGA-3' and reverse 5'-TTGAGGACCTCTGTGTATTTGTCAA-3']. An initial denaturing step of 95°C for 10 minutes was followed by 40 cycles of 95°C for 10 seconds; 10 seconds annealing at 68°C ; and elongation at 72°C for 10 seconds (GRPR and RPL13A) and 15 seconds (ERβcx). The fluorescence intensity of the double-strand specific SYBR GreenI was read at 72°C (GRPR and RLP13A) and 83°C (ERβcx) after the end of each extension step. In initial experiments, PCR products were purified and subjected to direct sequencing to verify amplification of the corresponding sequence. To determine the quantity of target cDNA transcript, cDNAs of known concentrations for target genes, and the housekeeping gene, RPL13A, were used to generate standard curves for qPCR [25-27]. The mRNA level in each case was represented as a ratio of RLP13A and was evaluated as a ratio (%) compared with that of each control [25-27]. Negative control experiments were done without cDNA substrate to examine the presence of exogenous contaminant DNA.

Immunoblotting. The cell protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt Protease Inhibitor Cocktail(Pierce Biotechnology), according to instruction manual. The concentration of the protein included in cell lysate was measured using Protein Assay Kit Wako (Wako Pure Chemical Industries, Osaka, Japan)). 20 μg of the protein (whole cell extracts) were subjected to SDS-PAGE(10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, England) using Mini Trans-Blot Cell and Power/Pac200 (Bio-Rad Laboratories, Hercules, CA). The blots were blocked in 5% non-fat dry skim milk for 1hr at room temperature, and were then incubated with a 1/1000 dilution of human ERβ antibody (GeneTex Inc., TX, USA), which detects both wtERβ and ERβcx, for overnight at 4 °C. After incubation with anti-mouse IgG horseradish peroxidase (GE Healthcare) for 1hr at room temperature, antibody-protein complexes on the blots were detected using ECL-plus western blotting detection reagents (GE Healthcare). The protein bands were visualized with LAS-1000 cooled CCD-camera chemiluminescent image analyzer (Fuji Photo Film Co, Tokyo, Japan).

Statistical Analysis. Statistical analysis was done using the StatView 5.0J software (SAS Institute, Cary, NC). In an analysis of the possible correlation between immunoreactivity and clinicopathologic variables, values for the patient age, serum PSA level, and LI for Ki-67, AR, ERβ and ERβcx were presented as the mean±95% confidence interval (95% CI). An association between GRPR immunoreactivity and each clinicopathological parameters were evaluated in a Spearman's rank correlation test. In the other studies, results were expressed as mean±SD, and analyzed by a Bonferroni test. In all statistical analysis, a p-*value*<0.05 was considered to indicate statistical significance.

Results

Immunnohistochemistry and correlation between GRPR immunoreactivity and clinicopathologic variables in 51 prostate carcinoma patients. GRPR immunoreactivity was detected in the cytoplasm of prostate carcinoma cells, while immunoreactivity of ERβcx was detectable in the nuclei of prostate carcinoma cells (Fig.1). Results of associations between GRPR immunoreactivity and clinicopathologic variables in 51 prostate carcinoma patients were summarized in Table1. The number of cases with immunoreactive GRPR in each group was summarized as follows: -, 28 cases (54.9%); +, 18 cases (35.3%); and ++, 5 cases (9.8%). The status of GRPR immunoreactivity was also significantly associated with Gleason score and ERβcx immunoreactivity of the cases, whereas there were no significant associations between GRPR and other clinicopathologic variables, including patients' age, serum PSA level, pathological stage, lymph node status, Ki-67 LI, AR LI, ERβ LI and NED status.

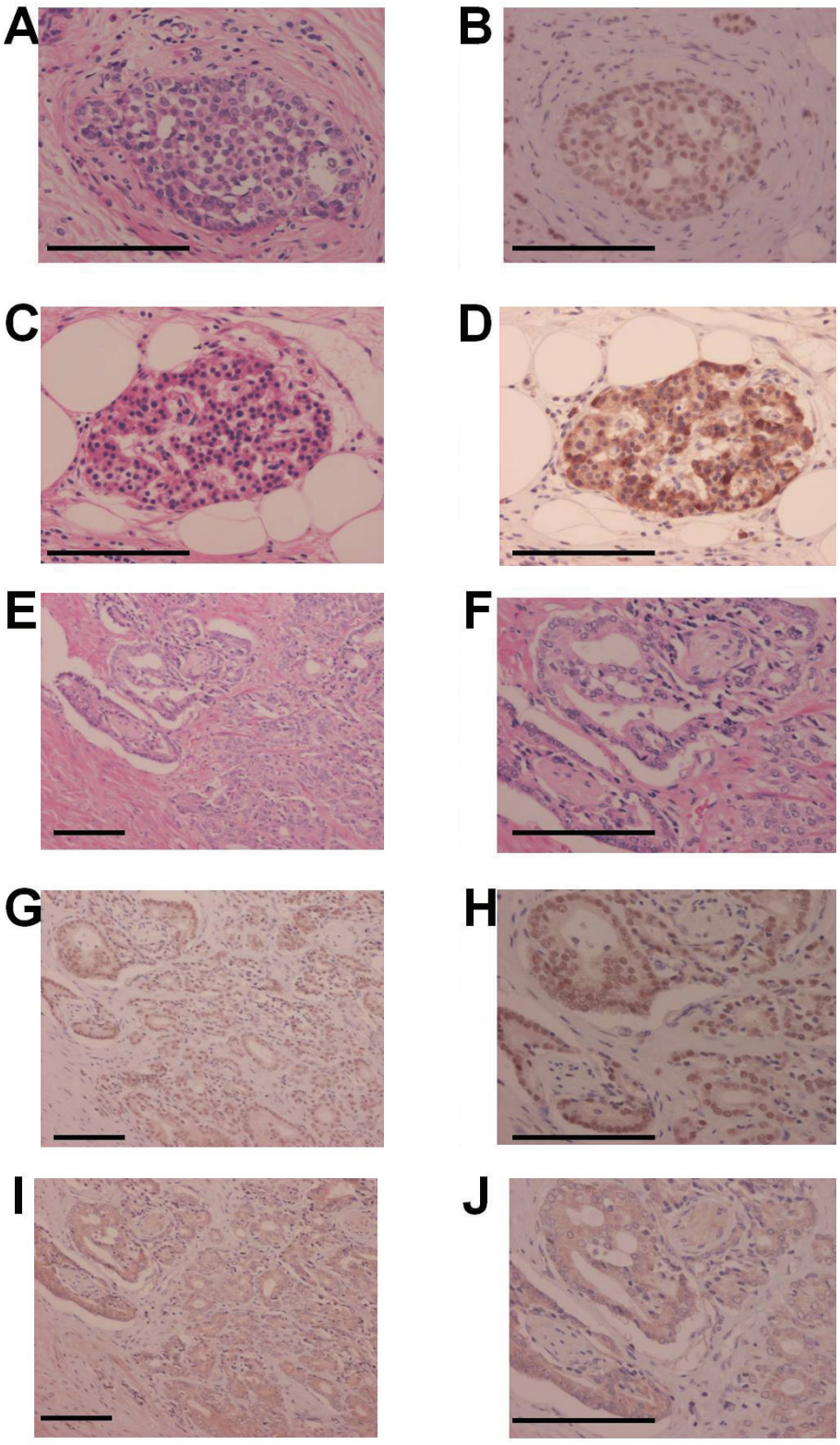

Figure 1. H&E staining (A) and immunohistochemistry of ERβcx (B) in human breast carcinoma (positive control). H&E staining (C) and immunohistochemistry of GRPR (D) in the islet of human pancreas (positive control). H&E staining (E, F), immunohistochemistry of ERβcx (G, H) and GRPR (I, J) in human prostate carcinoma. Immunoreactivity of ERβcx and GRPR were detected in the nuclei and cytoplasm of prostate carcinoma cells, respectively. Scale bar = 100μm

Table1. Association between GRPR immunoreactivity and clinicopathological parameters in 51 Prostate Carcinoma

	GRPR immunoreactivity						*P* value
	-		+		++		
	(*n*=28)		(*n*=18)		(*n*=5)		
Ages(years)	66.3 ± 2.2		64.1 ± 2.8		64.0 ± 8.3		0.1843
PSA(ng/mL)	11.9 ± 3.7		14.3 ± 7.0		16.8 ± 23.6		0.4075
Gleason score							
6	14	(27.5%)	0	(0.0%)	0	(0.0%)	
7	8	(15.7%)	11	(21.6%)	0	(0.0%)	
8	4	(7.8%)	4	(7.8%)	5	(9.8%)	
9	2	(3.9%)	3	(5.9%)	0	(0.0%)	**0.0003**
Stage							
pT2	14	(27.5%)	5	(9.8%)	2	(3.9%)	
pT3	14	(27.5%)	13	(25.5%)	3	(5.9%)	0.2156
Lymph node status							
Positive	1	(2.0%)	2	(3.9%)	0	(0.0%)	
Negative	27	(52.9%)	16	(31.4%)	5	(9.8%)	0.6045
Ki-67 LI (%)	7.3 ± 2.4		9.1 ± 2.5		6.0 ± 5.3		0.3431
AR LI (%)	71.6 ± 8.2		73.7 ± 13.1		81.2 ± 9.6		0.2946
ERβ LI (%)	39.1 ± 9.8		46.8 ± 16.5		40.0 ± 37.5		0.7310
ERβcx LI (%)	3.7 ± 2.2		11.9 ± 5.0		19.2 ± 16.9		**<0.0001**
NED status							
Positive	2	(3.9%)	4	(7.8%)	1	(2.0%)	
Negative	26	(51.0%)	14	(27.5%)	4	(7.8%)	0.3185

Data are presented as means±95% confidence interval. All other values represent the number of cases and percentage. Difference between each groups were determined by Spearman's rank correlation test. *P* values less than 0.05 were considered significant, and were represented in boldface.

Stable over-expression of ERβcx in PC-3 cells. ERβcx is expressed in PC-3 cell line at the basal level but its level of corresponding mRNA and protein were near or below the detection limit. PC-3 cells were therefore stably transfected with human ERβcx in order to clarify the function of ERβcx. Following the process of G-418 selection, the levels of ERβcx mRNA expression were evaluated using qPCR, and the clones that strongly expressed ERβcx mRNA were selected as positive clones in this experiment (Fig.2A). Expression of ERβcx protein was also evaluated using immunoblotting analysis (Fig.2B). We subsequently obtained two over expressing clones termed ERβcx-#7 and ERβcx-#8. Luciferase reporter gene assay was performed in order to further confirm the functionality of the ERβcx that was reported to block binding of ERs to estrogen responsive element and to function as dominant negative factors against ERs. ERβ expression vector and ERE-reporter vector were transiently transfected to Mock-#1 and ERβcx-#7 described above. ERE-dependent transactivation via ERβ by estradiol was significantly inhibited in ERβcx-#7 compared to Mock-#1 (Fig.2C).

GRPR mRNA expression in ERβcx over-expressing PC-3 cells using qPCR. We performed qPCR analysis for GRPR mRNA in the control strains transfected with empty vector, Mock-#1 and Mock-#2, and test strains over-expressing ERβcx, ERβcx-#7 and ERβcx-#8. Results of qPCR analysis were summarized in Fig.3A. The levels of GRPR mRNA expression in ERβcx-#7 and ERβcx-#8 were significantly higher (2 to 3fold) than that of Mock-#1 and Mock-#2.

ERβcx and GRPR mRNA expression in PC-3, DU-145 and LNCaP using qPCR. Results of qPCR analysis were summarized in Fig.3B. The amounts of ERβcx and GRPR mRNA expression were relatively higher in PC-3 than in DU-145 and LNCaP. The amounts of mRNA of these two genes were extremely low in androgen-dependent prostate cancer cell line LNCaP.

Discussion

GRPR expression was reported in human prostate cancer by qPCR analysis or receptor autoradiography using radiolabeled ligand for GRPR but GRPR immunolocalozation has not been reported in the literature to the best of our knowledge [17, 18]. Therefore, this is the first immunohistochemical study of GRPR in human prostate carcinoma. Results of our study demonstrated that 45% (+;35%, ++;10%) of the cases examined demonstrated positive immunoreactivity in the cytoplasm of the carcinoma cells. In addition, results of this our present study demonstrated statistically significant positive correlation between GRPR immunoreactivity and Gleason score, the most well-established prognostic factor

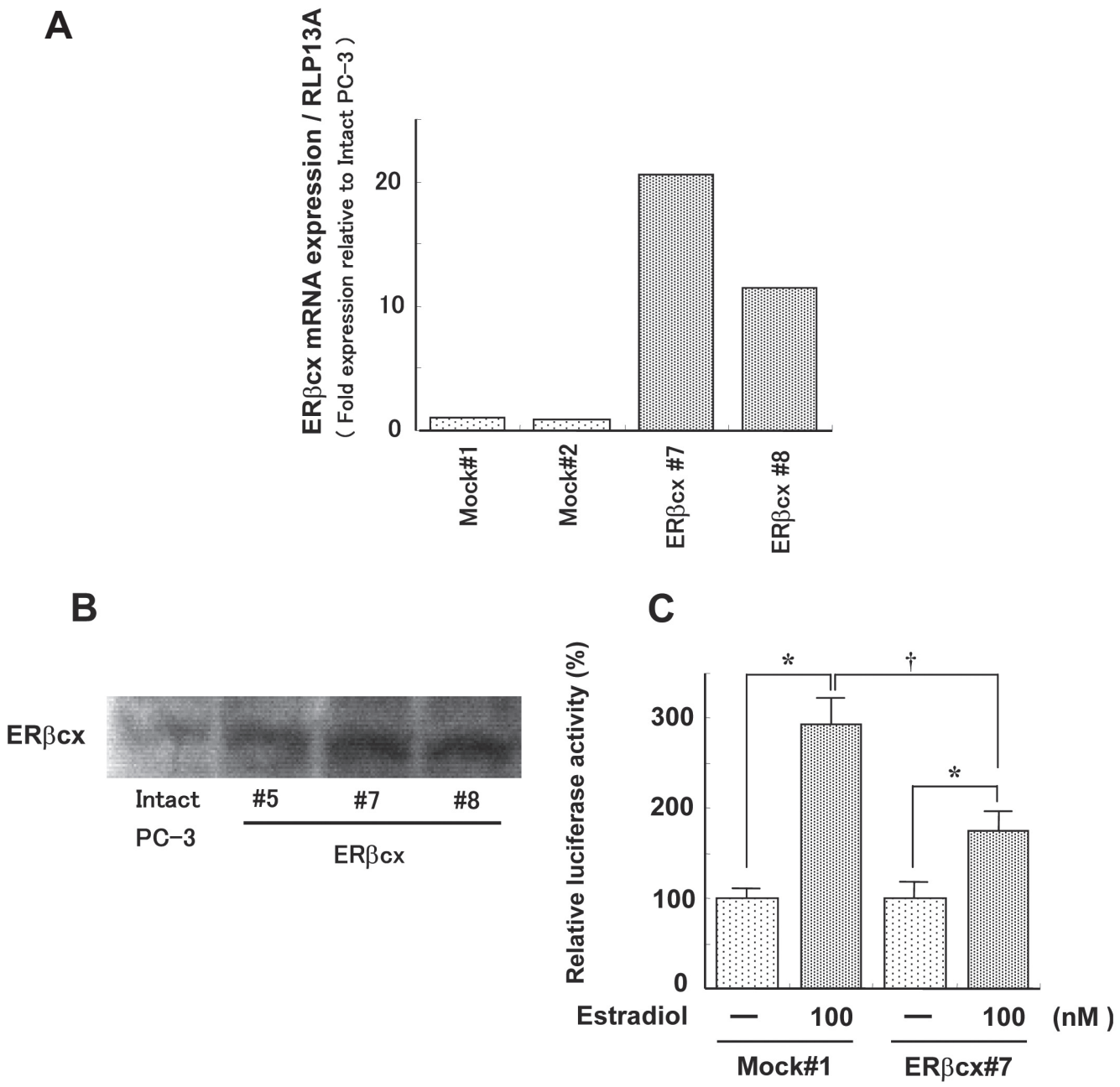

Figure 2. Isolation of transformants of PC-3 cells stably expressing ERβcx.
A: Relative expression of ERβcx in stably trasnfected with pRc/CMV or pRc/CMV-ERβcx vector in prostate cancer cell lines PC-3 studied by qPCR. High levels of ERβcx mRNA expression was detected (10 to 20fold) in ERβcx-#7 and ERβcx-#8 compared to Mock-#1 and Mock-#2.
B: Immunoblotting for ERβcx in stably trasnfected with pRc/CMV-ERβcx vector in prostate cancer cell lines PC-3
C: Results of dominant negative effect of ERβcx expressed in stably transfected PC-3. Mock-#1 and ERβcx-#7 were transiently transfected with pERE-Luc and pRc/CMV-ERβ plasmid, and treated with estradiol(100nM) for 24hr. The luciferase activity was evaluated as a ratio(%) compared to that of controls. Data are presented as mean±S.D.(n=3). $p<0.05$ vs each control(*) or induction ratio between each cell line(†).

in the patient with prostate carcinoma [28]. This correlation suggests also that the GRP secreted from neuroendocrne cells exert proliferation-stimulating effects via GRPR expressed in prostate cancer cells. Neuroendocrine differentiation is a common feature of adenocarcinoma of the prostate, and neuroendocrine differentiation is generally considered to be associated with aggressive biological behaviors in prostate carcinoma [29]. In addition, GRP/Bombesin antagonist was

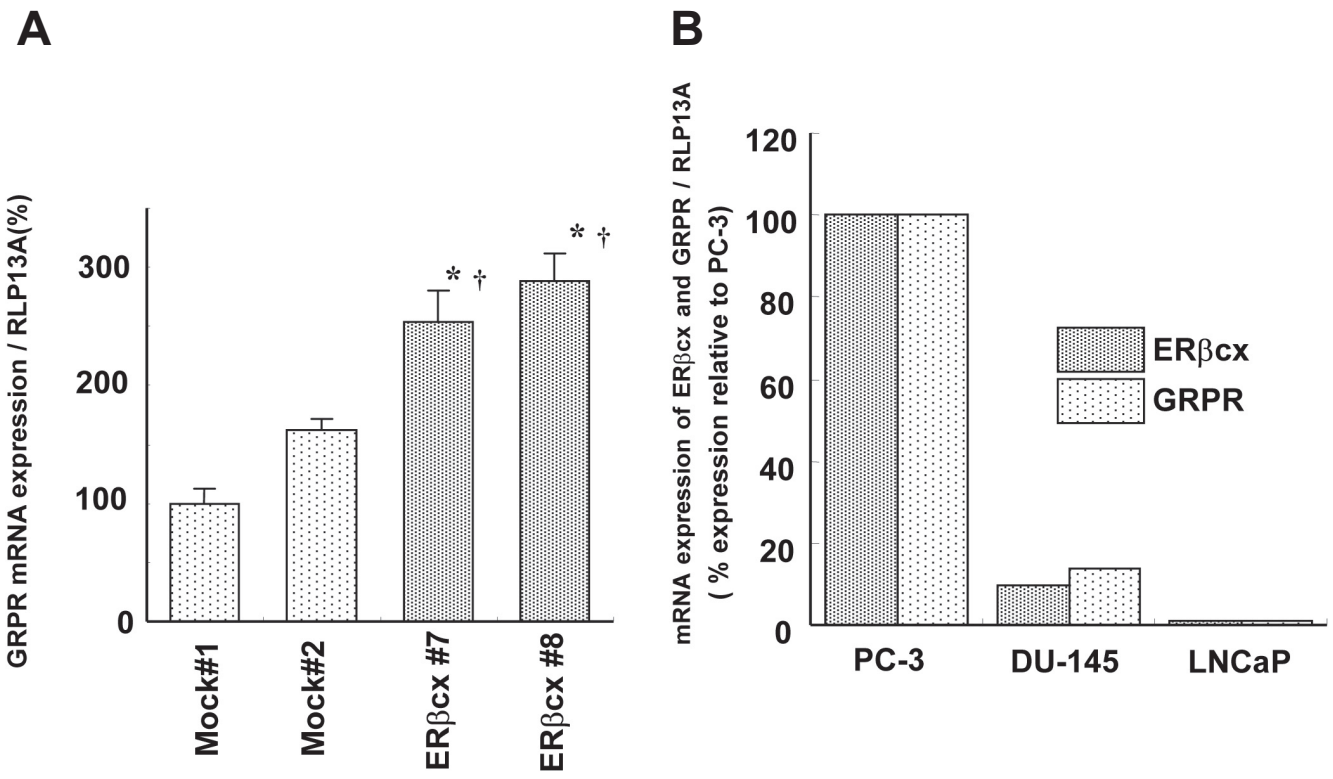

Figure 3. qPCR analysis of GRPR mRNA in ERβcx stably expressing PC-3 cells and human prostate cancer cell lines.
A: Relative expression of GRPR mRNA in stably trasnfected with pRc/CMV or pRc/CMV-ERβcx vector in prostate cancer cell lines PC-3 was analyzed by qPCR. 2 to 3 fold induction of GRPR mRNA was detected in ERβcx-#7 ERβcx-#8 compared to Mock-#1 and Mock-#2. The expression level of GRPR mRNA was evaluated as a ratio(%) compared to that of Mock-#1. Data are presented as mean±S.D.(n=3). $p<0.05$ vs Mock-#1(*) or Mock-#2(†).
B: Comparison of mRNA expression of ERβcx and GRPR in three prostate cancer cell lines, PC-3, DU-145 and LNCaP. Relative expression of ERβcx and GRPR mRNA in each cell lines were analyzed by qPCR. Expression pattern of ERβcx and GRPR were extremely resembled between three cell lines.

reported to significantly suppress the growth or proliferation of PC-3 tumors implanted into the nude mice [30]. Therefore, all of these data above and our present results suggested that the up-regulation of GRPR in prostate carcinoma cells may partially explain these clinical behaviors of prostate adenocarcinomas with neuroendocrine differentiation. However, GRPR immunoreactivity was not correlated with NED status in our study evaluated by chromogranine A and it awaits further investigations for clarification.

The significant correlations were also detected between the status of GRPR and immunoreactivity of estrogen receptor β (ERβ) isoform 2, called ERβcx in prostate carcinoma cases. ERβcx is the one of well-studied isoform of ERβ and expressed in several types of human malignancies including human prostate carcinoma [31-37]. ERβcx was also more abundant in high-grade prostate carcinomas than low grade ones [20]. In addition, clinical outcome of patients with higher ERβcx was worse than those with lower ERβcx in human prostate cancer [20]. Therefore, ERβcx is currently considered as one of the important prognostic factors in the patients with prostate carcinoma. These reports above and our finding suggest that the expression of GRPR could be, at least partially, mediated via ERβcx in direct or indirect manner in human prostate carcinoma cells and sequentially promote the progression of prostate cancer. Results of qPCR analysis in this our present study in which the amounts of ERβcx and GRPR mRNA were evaluated in three different prostate cancer cell lines also strongly supports the hypothesis discussed above. In addition, an induction of GRPR mRNA expression was confirmed in ERβcx over-expressing PC-3 cells. However, it awaits further investigations including some in vitro investigations to demonstrate the regulation of GRPR gene expression through ERβcx in detail.

In summary, we immunolocalized GRPR in 51 human prostate carcinomas and the status of GRPR was correlated with Gleson score and ERβcx LI. These findings also indicates that ERβcx contribute to prostate cancer development possibly through mediating GRPR expression in carcinoma cells.

Acknowledgments: We appreciate the skillful technical assistance of Mr. Katsuhiko Ono, Ms. Miki Mori and Ikumi Miura (Department of Pathology, Tohoku University School of Medicine). This work was supported by the grant from the Yamaguchi Endocrine Research Association.

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