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Abstract

The insulin-like growth factor I receptor (IGF-IR) is exceptionally overexpressed in many cervicalcancer-derived cell lines. It is postulated that a decrease of p53 protein levels due to human papillomavirus (HPV) infection may contribute to the up-regulation of IGF-IR expression in cervical cancer cells because transcription of IGF-IR is strictly down-regulated by p53. To evaluate this fact in clinical cervical cancer specimens, we checked the expression levels and activated status of IGF-IR by immunohistochemistry. Formalin-fixed and paraffin-embedded specimens obtained by conization or hysterectomy were stained with anti-IGF-IR and with an antibody recognizing phosphorylated tyrosine at its c-terminus. The expression levels of IGF-IR were significantly high in cervical intraepithelial neoplasia (CIN) III and invasive cancer specimens. Phosphorylation of IGF-IR was promoted in all CIN and invasive cancer specimens, and its intensity was related to the promotion of lesions. Interestingly, IGF-IR overexpression was missing in the basal layer of CIN I and II lesions, whereas it was evenly distributed in CIN III and invasive cancer lesions. This IGF-IR overexpression pattern may be utilized in the diagnosis of HPV infection status in CIN lesions.

KEYWORDS: insulin-like growth factor I receptor, cervical cancer, human papillomavirus, tyrosil phosphorylation

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Original Article

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The insulin-like growth factor I receptor (IGF-IR) is exceptionally overexpressed in many cervical-cancer-derived cell lines. It is postulated that a decrease of p53 protein levels due to human papillomavirus (HPV) infection may contribute to the up-regulation of IGF-IR expression in cervical cancer cells because transcription of IGF-IR is strictly down-regulated by p53. To evaluate this fact in clinical cervical cancer specimens, we checked the expression levels and activated status of IGF-IR by immunohistochemistry. Formalin-fixed and paraffin-embedded specimens obtained by conization or hysterectomy were stained with anti-IGF-IR and with an antibody recognizing phosphorylated tyrosine at its c-terminus. The expression levels of IGF-IR were significantly high in cervical intraepithelial neoplasia (CIN) III and invasive cancer specimens. Phosphorylation of IGF-IR was promoted in all CIN and invasive cancer specimens, and its intensity was related to the promotion of lesions. Interestingly, IGF-IR overexpression was missing in the basal layer of CIN I and II lesions, whereas it was evenly distributed in CIN III and invasive cancer lesions. This IGF-IR overexpression pattern may be utilized in the diagnosis of HPV infection status in CIN lesions.

Key words: insulin-like growth factor I receptor, cervical cancer, human papillomavirus, tyrosil phosphorylation

It is widely recognized that human papillomavirus (HPV) infection is the fundamental cause of cervical cancer [1, 2]. Previous reports revealed that almost 90% of cervical cancer patients were HPV-positive. The commonly used method for HPV detection is a polymerase-chain-reaction (PCR)-based amplification of the HPV L1 region by consensus primers. Therefore, the exact incidence of HPV infection, especially in invasive cervical cancer, might be higher because the L1 region of HPV is often disrupted upon integration of the HPV genome into the chromosomes. Actually, one report showed the incidence of HPV infection in invasive cervical cancer to be as high as 99.7% after re-evaluation by type-specific PCRs for the E7 gene, indicating that HPV plays an essential role in the carcinogenesis of cervical cancer [3]. The oncogenic functions of two HPV early proteins, E6 and E7, are well established: E6 binds to p53 by forming a trimeric complex with the cellular ubiquitin ligase E6-associated protein, and the p53 is rapidly degraded by proteasomes [4–6]; E7 binds to hypophosphorylated pRB, which is then rapidly degraded by proteasomes; thereby pRB constitutively releases transcriptional factor E2F [7–9]. The loss of function of these 2 major tumor suppressor gene products due to infection with HPV is a funda-

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mental cause of the carcinogenesis of cervical cancer. However, the whole mechanism of cervical cancer carcinogenesis after the inactivation of p53 and pRB remains to be elucidated.

Insulin-like growth factor I receptor (IGF-IR) is a tyrosine-kinase receptor which is located on the cellular membranes of normal cells as well as various cancer cells. Para-crine or auto-crine stimulation of IGF-IR with its ligand IGF-I, IGF-II, and insulin at supra-physiological concentrations leads to tyrosine auto-phosphorylation of IGF-IR, followed by trans-phosphorylation of its substrates IRS-1 and Shc, and tranduces various signals via PI3-kinase and MAP-kinase pathways. IGF-IR and IGF-I are essential for the establishment and maintenance of cellular transformation [10, 11]. Over-expression of IGF-IR and/or IGF-I has been reported to be associated with poor prognoses such as a high incidence of lymph node metastasis and a low survival rate in many kinds of malignancies [12, 13].

The expression of IGF-IR is usually not extremely high in many human cancer-derived cell lines, because the overexpression of IGF-IR may cause hyper-differentiation of the cells and may result in apoptosis. However, we previously reported that the transcription of IGF-IR was strictly down-regulated by the tumor suppressor gene product p53 [14]. Therefore, we postulated that the expression of IGF-IR in cervical cancer cells might be exceptionally high due to the decrease of p53 by means of the promoted proteolysis that is promoted after HPV infection.

The aim of the present study is to elucidate our hypothesis of IGF-IR up-regulation by HPV in human cervical cancer and pre-cancerous lesions, and to speculate on the role of IGF-IR in the carcinogenesis and progression of cervical cancer and also on its clinical application.

Materials and Methods

Patients and tissue samples. Included in this study are cervical intraepithelial neoplasia (CIN) patients and cervical cancer stage 0, stage I, and stage II patients (FIGO staging classification) who underwent conization or hysterectomy at Okayama University Hospital, Okayama, Japan, between January 2001 and December 2003. All subjects gave written informed consent. Basically, slices including the most advanced lesion within the surgical specimen were subjected to immunohistochemistry. As negative controls, cervical specimens from patients who underwent hysterectomy with benign diseases such as leiomyomas were used. Thirty consecutive individual specimens for each clinical stage pathologically shown to be CIN or squamous cell carcinomas were used for the study.

Cells. We employed R^- and R^+ cells as negative and positive controls for immunohistochemical evaluation of IGF-IR expression status. R^- cells are 3T3-like fibroblasts established from mouse embryo with targeted disruption of *IGF-I*, *IGF-II*, and *IGF-IR* genes [15]. R^+ cells are R^- cells stably transfected with human IGF-IR cDNA, and currently express 9×10^6 IGF-IRs per cell. HeLa S3, SiHa, and C33a cells are cell lines derived from human cervical carcinomas. These cell lines were obtained from the Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. CaOV-3 and OVCAR-3 cells are cell lines derived from human ovarian carcinoma purchased from American Type Culture Collection.

Western blot. The expression levels of IGF-IR in the cell lines were evaluated by western blot. Subconfluent cells were lysed in a lysis buffer (50 mM Hepes pH7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100) containing 100 mM NaF, 0.2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 1% phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin to detect the expression levels of IGF-IR. Thirty micrograms of whole cell lysates were separated on a 5–15% gradient SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained with anti-IGF-IR β-subunit antibody (C20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The blot was hybridized with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) and was developed using enhanced chemiluminescence detection reagents (Amersham Biosciences Corp., Piscataway, NJ, USA). To ascertain the sensitivities and specificities of the antibodies used in the study, total cell lysates collected from R^- and R^+ cells after stimulation with or without IGF-I (Invitrogen/Life Technologies, Inc., Rockville, MD, USA) were used for western blot analyses. Fifty micrograms of whole cell lysates were

separated and stained. Anti-IGF-IR β-subunit antibody or anti-PY1316 antibody [16] was used as the primary antibody. Anti-PY1316 antibody was raised from a synthetic peptide including phosphorylated tyrosine at amino acid position 1316.

Immunohistochemistry. Formalin-fixed and paraffin-embedded surgical specimens were stained with anti-IGF-IR β-subunit antibody (C-20, Santa Cruz Biotechnology, Inc.) or with anti-PY1316 antibody. Sections of 4 μm in thickness were deparaffinized, rehydrated with xylene and ethanol, and blocked with methanol with 3% hydrogen peroxide. Sections were incubated with primary antibodies (1: 500 dilution in PBS for anti-IGF-IRβ, and 1: 300 in PBS for anti-PY1316) for 2 h at room temperature. Then sections were stained with Histofine Simple Stain MAX-PO (MULTI) and Simple Stain DAB Solution (Nichirei Bioscience, Tokyo, Japan) according to the manufacturer's protocol. The slides were counterstained with hematoxylin and used for the study. We also used R^- cells as negative controls and R^+ cells as positive controls, respectively, in each staining. R^- cells or R^+ cells were cultured on a Lab-Tek™ Chamber Slide™ System (Thermo Fisher Scientific Inc., MA, USA), fixed by 1% formalin and stained in the same manner. To evaluate tyrosil phosphorylation of IGF-IR, R^- cells or R^+ cells were treated as described above and fixed immediately with formalin.

Staining evaluation. The intensity of staining was graded as 0 (negative: no staining is present or partial membrane staining in $<10\%$ of malignant or dysplastic cells), 1+ (light staining: weak/barely perceptible membrane staining in $<10\%$ of malignant or dysplastic cells, but the cells are only stained in part of their membrane), 2+ (moderate staining: weak to moderate complete membrane staining is present in $>10\%$ of malignant or dysplastic cells), or 3+ (heavy staining: moderate to strong complete staining in $>10\%$ of malignant or dysplastic cells). Microscopic analyses were evaluated independently by the first 2 authors in comparison with negative and positive controls in the same immunohistochemistry series. Final evaluations of ambiguous cases (less than 20% of the samples) were made on a conference microscope with other authors.

Statistical analyses. Univariate analysis with the *t*-test was performed using Stat-View 5.0 software (Abacus Concepts, Berkeley, CA, USA), and probability values less than 0.01 were considered statistically significant.

Results

IGF-IR expression levels in cervical cancer cell lines. We checked the expression levels of IGF-IR in cervical-cancer-derived cell lines to test our hypothesis described above. IGF-IR was exceptionally overexpressed in Hela S3 cells, harboring ~50 copies of HPV. IGF-IR expression was also high in SiHa cells, harboring 1–2 copies of HPV, but IGF-IR expression in HPV-negative C33a cells was very low as in HPV-negative ovarian cancer cells (Fig. 1).

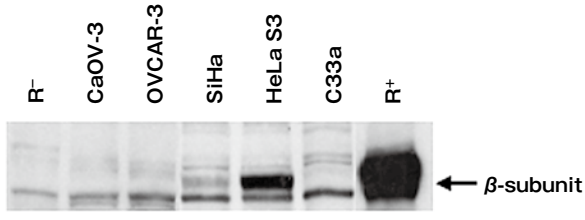

Fig. 1 IGF-IR overexpression in cervical cancer cell lines. Thirty micrograms of total cell lysates from representative cervical cancer cell lines were separated in SDS-PAGE and stained with anti-IGF-IR β-subunit. IGF-IR is overexpressed in HPV-positive cervical cell lines in comparison to ovarian cancer cell lines and HPV-negative cervical cancer cell line C33A.

Evaluation of the antibodies by western blot. The sensitivity and specificity of the 2 antibodies used for the study were evaluated by western blot. Total cell lysates from R^- cells and R^+ cells cultured in DMEM supplemented with 10% FBS or total cell lysates collected from R^- cells and R^+ cells with or without IGF-I stimulation were used. Western blots with antibody against the β-subunit of IGF-IR (C20) (Fig. 2A) and with the antibody against tyrosil-phosphorylated IGF-IR (PY1316) (Fig. 2B) proved that both of these antibodies were highly sensitive and specific.

Evaluation of the antibodies by immunohistochemistry. We then checked the sensitivity and specificity of the 2 antibodies by immunohistochemical staining. Again, R^- cells and R^+ cells were cultured on glass slides and stained with these antibodies. R^-

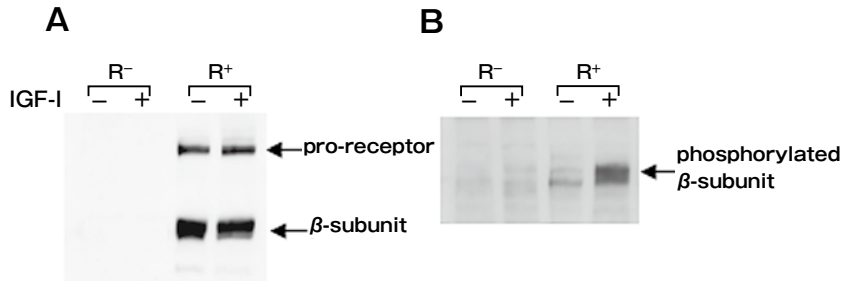

Fig. 2 Western blot of IGF-IR expression and its phosphorylation.
Expression of IGF-IR and its tyrosil-phosphorylation were evaluated by Western blot. Fifty micrograms of cell lysates from R^- or R^+ cells with or without stimulation with IGF-I were electrophoresed and stained with antibodies against IGF-IR β-subunit (**A**) or the phosphorylated c-terminus of the IGF-IR (**B**).

cells with or without IGF-I stimulation were not stained with these antibodies, showing high specificities of the 2 antibodies (Fig. 3). A basal level of IGF-IR tyrosil-phosphorylation in R^+ cells after starvation was detected by immunohistochemistry, suggesting a sufficient sensitivity of PY1316 in immunohistochemistry, and phosphorylated IGF-IR was detected as expected in stimulated R^+ cells (Fig. 3).

Immunohistochemical analyses of surgical specimens. Surgical specimens from CIN I, CIN II, CIN III, and squamous cell carcinomas from stage I and II cervical cancer patients were stained with anti-IGF-IRβ or anti-phosphorylated IGF-IR antibodies. Representative pictures at each clinical stage are shown in Fig. 4. IGF-IR expression was partly positive in CIN I and CIN II lesions in comparison with normal cervical epithelia. In CIN III and invasive cervical cancer specimens, IGF-IR expression became more intense, and its expression was evenly distributed within those lesions.

Phosphorylation of IGF-IR was hardly visible in CIN I and CIN II specimens as well as in normal cervical epithelium. The intensity of IGF-IR phosphorylation was related to the promotion of lesions among CIN III and invasive cancer specimens. Staining intensity by immunohistochemistry was graded from 0 to 3+ according to the criteria shown in the Material and Methods. This staining evaluation is summarized in Table 1 and Table 2, and the mean and standard deviation of the staining intensity of each stage are shown in Fig. 5. The expression levels of IGF-IR in CIN III and invasive cervical cancer lesions were significantly higher in comparison with normal cervical epithelia (Fig. 5A). The phosphorylation status of IGF-IR in all stages was higher in comparison with normal cervical epithelia, and its intensity increased in accordance with the progression of the disease (Fig. 5B).

It is of interest, as shown in a CIN II specimen stained with anti-IGF-IRβ, that the expression of IGF-IR appears from intermediate cells to superficial cells in the cervical epithelium. In contrast, IGF-IR expression in basal and para-basal cells is very faint (Fig. 6A). In CIN III lesions, the IGF-IR expression appears evenly in every layer of the epithelium, and it becomes more intense (Fig. 6B). So we re-evaluated our CIN I, CIN II, CIN III and stage Ia specimens that retained enough intact basal cell area to observe, and checked whether IGF-IR staining was excluded within basal and para-basal cells or not. The percentage of this basal and para-basal cell overexpression of IGF-IR is shown in Table 3. Basal cell overexpression of IGF-IR in CIN III lesions was more common in comparison with CIN I and CIN II lesions.

Discussion

The present study was intended to evaluate the expression levels and activated status of IGF-IR in human cervical cancer specimens to elucidate how IGF-I axis play a role in the initiation and progression of cervical cancer with malignant-type HPVs. Although we did not check investigate the prevalence or genotypes of HPV in the present study, previous reports have already proven that almost all invasive cervical cancer contains malignant-type HPVs [3].

Immunohistochemistry with anti-IGF-IRβ antibody revealed that the expression levels of IGF-IR in CIN

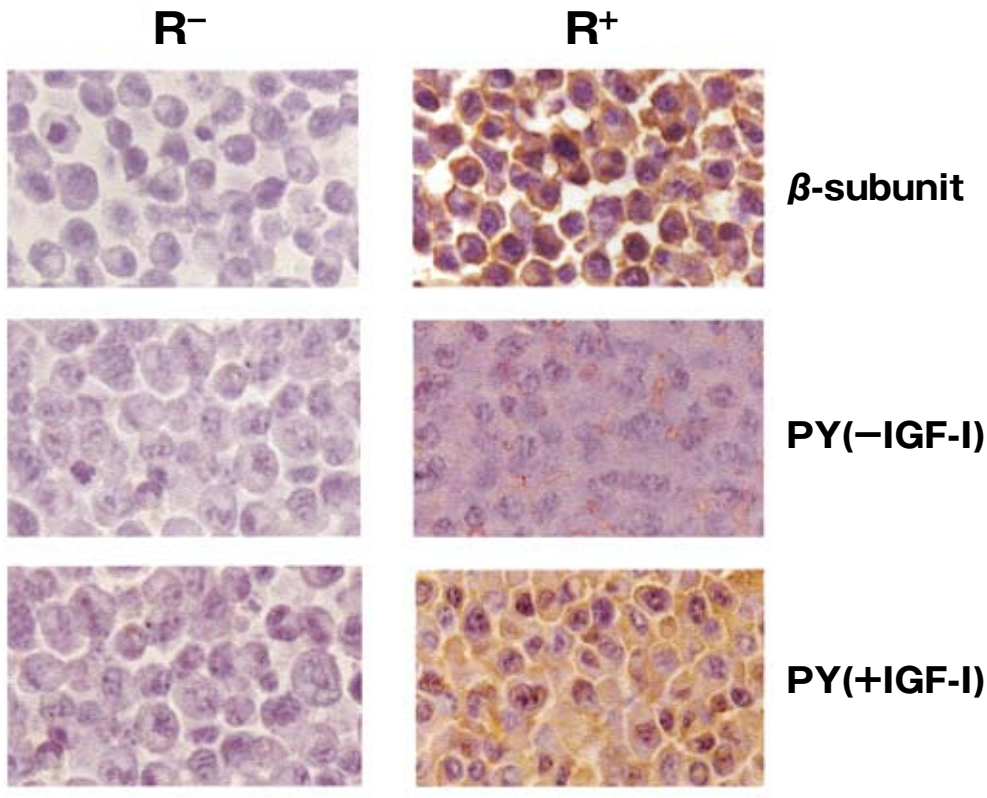

Fig. 3 Immunohistochemical staining of R^- and R^+ cells.
R^- cells and R^+ cells were stained with anti-IGF-IRβ antibody (upper panel). Also, R^- cells and R^+ cells were stimulated with (lower panel) or without (middle panel) IGF-I and stained with anti-phosphorylated IGF-IR antibody.

Table 1 IGF-IR expression

Staining intensity	0	1+	2+	3+
Normal	18	12	0	0
CIN I	8	20	2	0
CIN II	7	19	4	0
CIN III	5	13	10	2
Stage Ia	7	14	5	4
Stage Ib	5	10	10	5
Stage IIb	0	11	13	6

Table 2 IGF-IR phosphorylation

Staining intensity	0	1+	2+	3+
Normal	28	2	0	0
CIN I	27	3	0	0
CIN II	23	7	0	0
CIN III	16	10	4	0
Stage Ia	7	17	6	0
Stage Ib	0	16	9	5
Stage IIb	0	8	10	12

III lesions and invasive squamous cell carcinoma lesions were significantly higher in comparison with those of normal cervical epithelial cells (Fig. 5A). IGF-IR expression in CIN I and CIN II lesions, exclusively within intermediate cells, was intensified, although it was not statistically significant. These limited overexpressions of IGF-IR in CIN I and CIN II lesions were compatible with the localization of E6 protein expression in HPV-infected cervical epithelium. CIN I and CIN II lesions reportedly display viral expression patterns suggestive of productive viral infection [17–19]. This viral replication is dependent on the differentiation process of the infected epithelium. Viral activity in infected basal cells is low, and episomal HPVs in basal cells are co-replicated with the genome of the host cells.

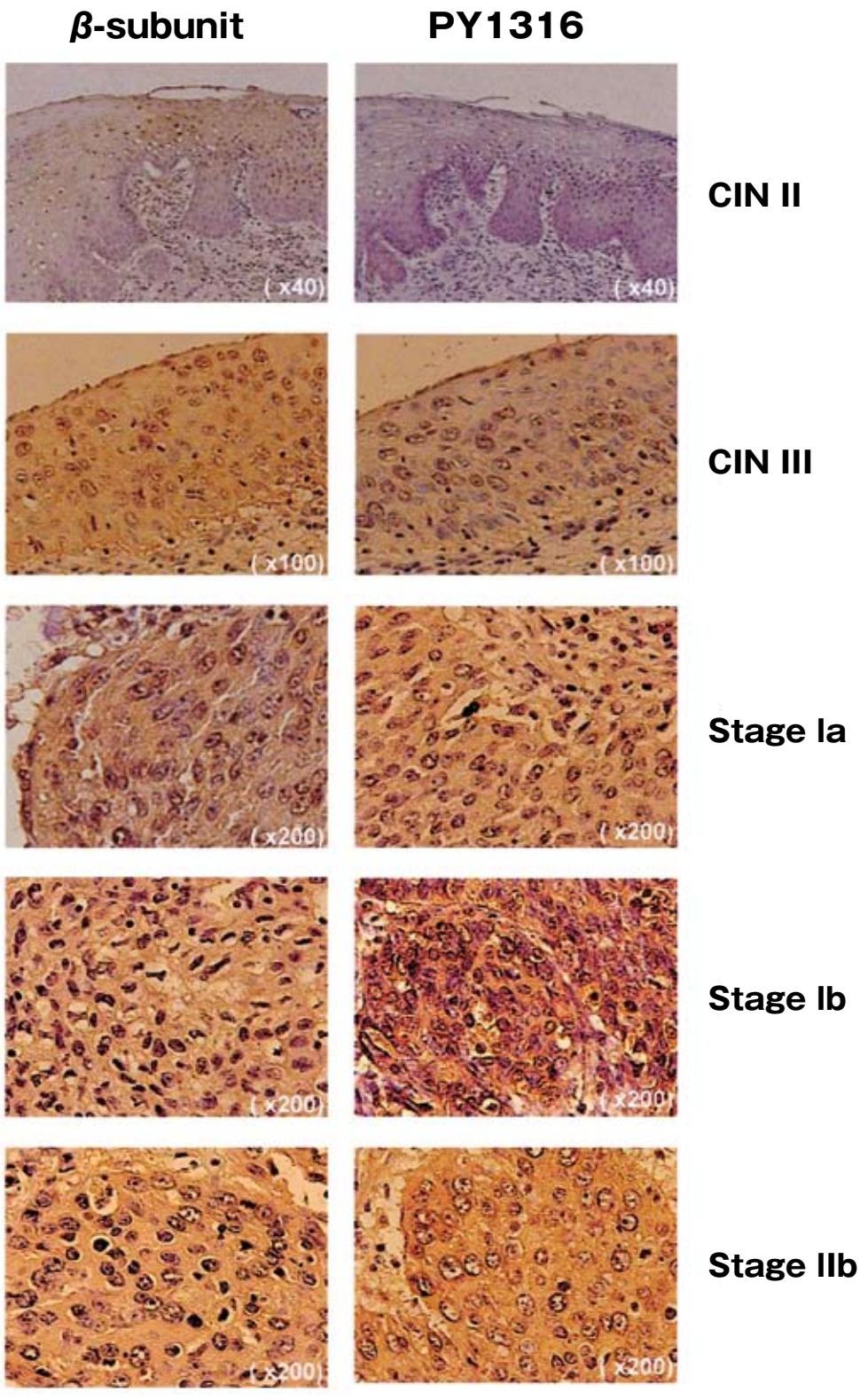

Fig. 4 Representative immunohistochemical staining with anti-IGF-IRβ and anti-phosphorylated IGF-IR (PY1316).

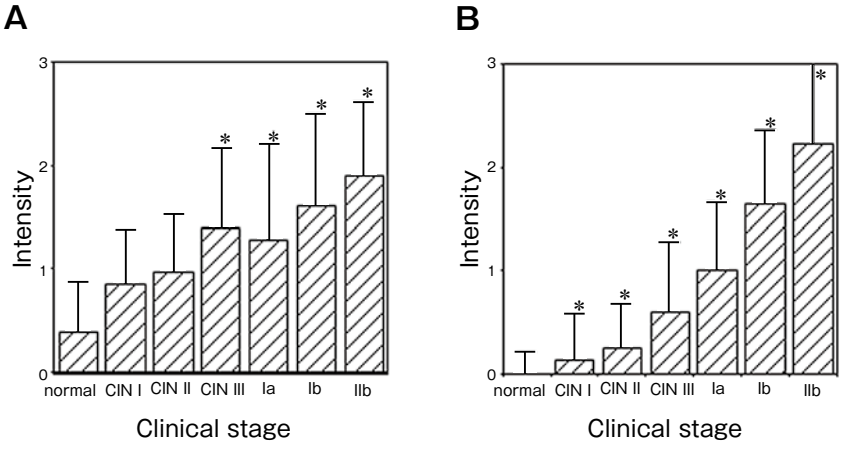

Fig. 5 Immunohistochemical staining intensity.
Surgical specimens were stained with anti-IGF-IRβ (Panel A) and anti-phosphorylated IGF-IR (Panel B). Staining intensity was graded as described in Materials and Methods, and the means and standard deviations from 30 consecutive samples were compared.
*: $p < 0.01$ against normal epithelium.

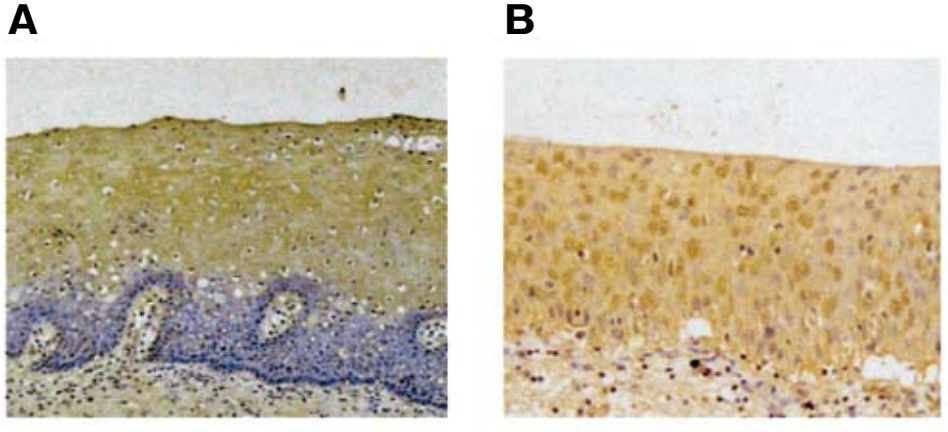

Fig. 6 IGF-IR overexpression in basal and para-basal cells.
A, A representative CIN II lesion expressing IGF-IR especially in intermediate cells and lacking its expression in basal and para-basal cells; **B**, A representative CIN III lesion whose IGF-IR expression is evenly distributed within all epithelial layers including basal and para-basal cells.

Table 3 IGF-IR overexpression in basal cells

	Positive	Negative	Positive rate (%)
Normal	0	10	0
CIN I	1	11	8.3
CIN II	3	9	25.0
CIN III	8	3	72.7
Stage Ia	12	0	100

However, viral transcription including that of E6 and E7 is markedly increased, especially in intermediate cells, upon differentiation of the epithelium [19]. The distribution of IGF-IR-overexpression in CIN I and CIN II lesions shown in the present study was co-located with that of HPV E6 oncoprotein expression during viral replication within these early infected lesions. The expression of E6 in episomal HPV is lower than that of deregulated integrated HPV, but the copy number of infected HPV in episome is usually pretty high in early CIN lesions. The

distribution pattern of IGF-IR overexpression in CIN I and CIN II in the present study (Fig. 6A) may also support this mechanism.

In contrast, IGF-IR-overexpression in CIN III and invasive cervical cancer lesions were evenly distributed within the epithelium (Fig. 4), indicating the deregulated expression of E6 within these lesions. HPV genomes in high-grade CIN lesions and cervical carcinoma lesions are often integrated in the host chromosomes [20]. *E2* gene, a repressor of *E6* and *E7* transcription, is often disrupted upon integration of the HPV genome into the chromosomes, resulting in the deregulated strong transcription of *E6* and *E7* regardless of the replication of the host cells [21]. Actually, our immunohistochemical evaluation showed that the expression levels of IGF-IR were significantly higher in CIN III and squamous cell carcinomas where the integration of HPV was likely. The IGF-IR-overexpression pattern shown in the present study was compatible with our hypothesis of HPV E6-induced transcriptional up-regulation of IGF-IR through p53 inactivation. The deregulated high expression of E6 due to the integration of the HPV genome into the host chromosomes may be the cause of the overexpression of IGF-IR in CIN III and cervical squamous cell carcinoma lesions.

The detection of IGF-IR overexpression and its localization may be utilized to distinguish viral replication in early CIN lesions and deregulated viral malignant transformation leading to carcinogenesis in late CIN lesions. Our immunohistochemical results indicated that the incidence of IGF-IR staining in basal cells was higher in CIN III lesions than CIN I and CIN II lesions (Table 3). Although the total number was small, 25% of CIN II lesions demonstrated IGF-IR overexpression in basal cells, suggesting a possible integration of HPV. Because conventional pathological diagnosis is based on morphology, it does not always reflect the status of HPV infection. It is also possible that a small number of CIN II lesions already possess integrated HPVs. This method may predict the progression of the disease more precisely than morphological change of the cervical epithelium.

Although a similar strategy to detect HPV infection by immunohistochemical detection of the up-regulated $p16^{INK4A}$, whose transcription is down-regulated by pRB, was previously reported [22, 23], the localization of $p16^{INK4A}$ within the epithelia has not been mentioned. Since immunohistochemical evaluation of IGF-IR distribution as well as $p16^{INK4A}$ distribution within CIN lesions seems to be an alternative method to verify the integration status of HPV, these methods can be an easier, time-saving and less expensive way to predict the prognosis of CIN lesions rather than conventional methods like fluorescent *in situ* hybridization.

Aside from the IGF-IR distribution pattern within CIN lesions, we could not see any correlations between IGF-IR overexpression and poor prognostic factors, such as lymphnode metastasis, lymph-vascular space involvement, and deep stromal invasion. The usage of IGF-IR overexpression as a prognostic factor for cervical cancer should be further elucidated in future analyses involving more samples.

On the other hand, the tyrosil autophosphorylation status of IGF-IR was significantly increased in all CIN and invasive squamous cell carcinoma lesions in comparison with those of normal epithelium (Fig. 5B). Judging from the staining intensity with the tyrosine-phosphorylation-specific antibody, the phosphorylation intensity was related to the promotion of the disease (Fig. 5B). There was a discrepancy between IGF-IR expression levels and its activated status. The expression levels of IGF-IR are enhanced by the deregulated E6 expression after the integration of HPV, but the activation of IGF-IR is provided by the abundant paracrine stimulation with its ligand IGF-I (and IGF-II) from surrounding cancer cells as well as stromal cells in advanced bulky lesions.

In the present study, we confirmed the overexpression of IGF-IR in CIN III lesions and invasive squamous cell carcinoma lesions, and this overexpression could also be observed exclusively in intermediate cells in CIN I and CIN II lesions. Since the overexpression of IGF-IR is likely to co-localize with deregulated E6 overexpression in CIN and squamous cervical cancer specimens, these findings may support our hypothesis of IGF-IR up-regulation by HPV E6 through deregulation from p53-induced transcriptional down-regulation of IGF-IR. We also confirmed that phosphorylation of IGF-IR increased according to the progression of the disease. Along with the up-regulation of IGF-IR expression by HPV E6, this increased phosphorylation of IGF-IR activates IGF-I axis signal transduction pathways, and possibly plays an important role in the progression of the disease via sending

strong cell survival signals.

In conclusion, results from the immunohistochemical evaluation of IGF-IR status in human specimens reconfirmed our hypothesis that HPV E6 induced up-regulation of IGF-IR by means of deregulation from the p53 negative loop. This up-regulation as well as increased tyrosil autophosphorylation of IGF-IR from CIN III lesions may contribute to the initiation and progression of cervical cancer. In addition, evaluation of the localization of IGF-IR overexpression in the cervical epithelium may be useful for distinguishing viral reproduction and malignant transformation in CIN lesions.

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