Retinoic Acid Receptor-β Expression in Stage I Non-Small Cell Lung Cancer and Adjacent Normal Appearing Bronchial Epithelium

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Retinoic acid receptor-β (RAR-β) is induced by and mediates the growth-inhibitory and apoptotic effects of retinoic acid (RA), suggesting that loss of RAR-β expression may be one of the critical events involved in the carcinogenesis/progression of non-small cell lung cancer (NSCLC) and in the responsiveness to retinoid chemotherapy. However, recent contradictory reports that the expression of RAR-β is associated with poor clinical outcome, and the fact that treatment of serum-deprived type 2 alveolar cells with RA leads to a stimulation of cell proliferation, require the verification of RAR-β as a biomarker of chemoprevention or prognosis. The expression status of RAR-β in cancer cells and adjacent normal appearing bronchial epithelium from 39 patients, diagnosed as stage I NSCLC and undergone a curative lung resection, was analyzed in paraffin-embedded tissue sections by IHC staining. The normal appearing bronchial epithelium of 14 out of 33 (42.4%) specimens expressed RAR-β, whereas 22 out of the 39 (56.4%) stage I NSCLC specimens expressed RAR-β. RAR-β was more frequently expressed in the adenocarcinoma (72.7%) than in the squamous cell carcinoma (31.3%) (p=0.026). Neither the expression status in normal appearing adjacent tissue nor that in the tumor tissue had prognostic implications. The higher expression of RAR-β in cancer tissue, the focal and uneven distribution in normal appearing adjacent bronchial epithelium, and inconsistency with the corresponding tumor tissue, suggest that the expression status of RAR-β as a biomarker for chemo-prevention/early diagnosis or the prognosis of NSCLC requires further consideration.

Key Words: Biomarker, chemoprevention, retinoic acids, retinoic acid receptor-β, non-small cell lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer death in men and women throughout the world, including the United States. Despite of intensive efforts to control lung-cancer mortality with standard surgery, radiation and chemotherapy, it was estimated that the United States had experienced 157,400 lung cancer deaths in 2001.[1] The 7% of 5-year survival rate in 1970, has marginally improved to 14%. Over 1,000,000 new cases of lung cancer are diagnosed worldwide each year.[2] However, 20% of stage I and 30% of stage II patients develop recurrent cancer, which is often incurable at the time of discovery. This grim overview indicates the urgent need for the elucidation of lung cancer carcinogenesis, as well as new approaches for its prevention/treatment, such as chemoprevention.

The molecules that participate in lung development and homeostasis are the retinoids. In vitro model of fetal lung development, the addition of retinoic acid (RA) has been associated with changes in the pattern of lung development, as characterized by increased growth of proximal airways.[3] Massaro and Massaro reported that

treatment with RA prevented the decrease in the number of alveoli caused by dexamethasone in newborn rats.[4] In infants, some clinical trials have reported decreases in the incidence and severity of bronchopulmonary dysplasia (BPD) with supplemental vitamin A.[5] Animals deprived of vitamin A demonstrated the same morphological alteration of the lungs as dead BDP children, with the development of squamous metaplasia in the tracheobronchial epithelium and the loss of ciliated cells. These studies suggest that retinoids play roles in postnatal lung growth and in the processes of lung repair following injury, which maintains lung integrity.[6]

The molecular actions of retinoids, important molecules for lung growth and homeostasis, are primarily mediated by their nuclear receptors (RAR-α, β and γ, and the retinoid X receptors-α, β and γ), which function as liganded transcription factors.[7] RAR-β, which is considered to be lost in the majority of non-small cell lung cancer (NSCLC) cell lines and tissues, is induced by and mediates the growth-inhibitory and apoptotic effects of RA,[8,9] suggesting that loss of RAR-β expression may be one of the critical events involved in the carcinogenesis/progression of lung cancer and in the responsiveness of lung cancer cells to retinoid chemotherapy. Loss of RAR-β expression in the bronchial epithelium is considered as a biomarker of preneoplasia in the field of chemoprevention; retinoids can restore the expression of this receptor and, presumably, halt the progression of carcinogenesis.[10] However, the presence of RA receptors and RA-binding proteins in the alveolar epithelium, and the fact that treatment of serum-deprived type 2 alveolar cells with RA led to the stimulation of cell proliferation in a dose-dependent manner, suggest that RA and its receptors, including RAR-β, may be associated with type 2 alveolar cell proliferation and differentiation.[7] Another chemopreventive study, examining bronchial biopsy specimens from heavy cigarette smokers using in situ hybridization (ISH), indicates that RAR-β expression may be an indicator of increased risk of lung cancer in heavy smokers.[11]

These contradictory theories led us to investigate the expression status of RAR-β in primary NSCLC and adjacent normal appearing lung tissue to see whether RAR-β is an appropriate surrogate biomarker in NSCLC chemoprevention fields. The clinical features associated with the down regulation of RAR-β expression in stage I NSCLC patients was also studied using immunohistochemical staining.

MATERIALS AND METHODS

Study population

Thirty-nine tissue specimens were obtained from patients whose pathological diagnoses were confirmed as stage I NSCLC and who had undergone a curative surgical removal of a primary lesion between 1994 and 1998. These specimens were randomly selected from the tissue bank at Yonsei University College of Medicine, Severance Hospital, Seoul, Korea. None of the patients had undergone either radiotherapy or chemotherapy before or after surgery until the disease had recurred. The histological diagnoses and classifications of the tumors were based on the World Health Organization (WHO) criteria. The post-surgical, pathological tumor-nodal-metastatic (TNM) stage of each malignancy was determined according to the guidelines of the American Joint Committee on Cancer. As shown in Table 1, the patients had a mean age of 59.8 ± 10.66 years, ranging from 32 to 76. Twenty-six (67%) and 13 (33%) of the patients were men and women, respectively. Twenty-three (59%) were smokers, 22 (56%) had adenocarcinoma, 16 (41%) squamous cell carcinoma, and 1 (3%) large cell carcinoma.

IHC for RAR-β

All of the sections were deparaffinized in a series of xylene baths and then rehydrated using graded alcohol series. Following deparaffinization, the slides were heated in an autoclave at 120℃ for 5 min, in a 10 mM citric acid buffer (pH 6.0). The sections were then immersed in methanol, containing 0.3% hydrogen peroxidase, for 15 min to block the endogenous peroxidase activity, and then incubated in a 2.5% blocking serum to reduce nonspecific binding. The sections were incubated overnight at 4℃ with primary anti-RAR-β polyclonal antibody (1/100 dilution; Santa

Table 1. Clinical and Pathological Characteristics of the Stage I NSCLC Patients according to Expression Status of RAR-β

| | | RAR-β expression in tumor tissue | | *p*-value |
		Expression (n=22)	No expression (n=17)	
Age		57.3 ± 11.36	63.1 ± 8.97	0.093
Gender	Male	13 (59%)	13 (76%)	0.318
	Female	9 (41%)	4 (24%)	
Smoking status	Smoker	11 (50%)	12 (70%)	0.285
	Non-smoker	6 (27%)	4 (24%)	
	Unknown	5 (23%)	1 (6%)	
TNM stage	T1N0M0	9 (41%)	5 (30%)	0.344
	T2N0M0	13 (59%)	12 (70%)	
Pathology	Squamous cell carcinoma	5 (23%)	11 (66%)	0.026
	Adenocarcinoma	16 (73%)	6 (34%)	
	Large cell carcinoma	1 (4%)	0 (0%)	
Histologic grade	Well differentiated	7 (32%)	5 (30%)	0.769
	Moderately differentiated	5 (23%)	6 (34%)	
	Poorly differentiated	7 (32%)	5 (30%)	
	Unclassified	3 (13%)	1 (6%)	

Cruz Biotechnology, Santa Cruz, CA, USA) and then processed using avidin-biotin IHC techniques. Representative areas of each cancer tissue and corresponding normal appearing bronchial epithelium, affixed to the tumor specimen in the same slide, were selected, and the cells counted in at least four fields (at × 200). All of the slides were independently evaluated and scored, by pathologists who were blind to the clinical information of the subjects, for the percentage of positive cells. The RAR-β immunoreactivities were classified as negative (less than 5% of counted cells expressed RAR-β), trace (5 - 10% expressed RAR-β), 1 + (10 - 50% expressed RAR-β) and 2 + (more than 50% expressed RAR-β). A 10% labeling index was applied as a cutoff point for the loss of RAR-β expression.

Statistical analysis

In a univariate analysis, independent sample *t*-tests and χ^2-tests were used for continuous and categorical variables, respectively. The Kaplan-Meier estimator was used to compute the survival probability as a function of time. The log-rank test was used to compare the survival times between the patient groups. Overall, disease-specific, event-free, and disease-free survival times were also analyzed. Cox regression was used to model the risk of the loss of expression on the survival time, with adjustment for clinical and histopathological parameters (age, sex, tumor histology subgroup, grade of differentiation, and smoking status). All of the statistical tests were two-tailed; a $p < 0.05$ was considered statistically significant.

RESULTS

Expression of RAR-β in adjacent normal appearing lung tissues

The human NSCLC cell line, A549, which exhibited strong RAR-β expression by Western blot analysis, was used as a positive control, and H1299 cells, which did not show RAR-β expression, were used as negative controls (data not shown). The staining for RAR-β was prominent in the cytoplasm of a portion of the histologi-

cally normal appearing bronchial epithelial cells
(Fig. 1). The intensity of staining was strong and
distinct and localized in the perinuclear area.
Frequent staining was observed in the perinuclear
area of basal and parabasal cells and that in
ciliated cells was not uncommon. There was a
significant difference in the percentage of stained
cells, not only between bronchial epithelia in a
slide, but also between slides. The status of the
RAR-β expression could not be evaluated in 6 out
of 39 (15.4%) slides due to the lack of bronchial
epithelium in those slides. Normal appearing
adjacent bronchial epithelial cells in 19 out of 33
(57.6%) slides expressed no RAR-β at all. In the
remaining slides, the normal appearing adjacent
bronchial epithelium of 2 slides (6.1%) were
scored as 'trace', 9 (27.3%) as '1+' and 3 (9.1%)
as '2+'.

Expression of RAR-β in stage I NSCLC tissues

The expression of RAR-β in stage I NSCLC
cells was localized mainly in the perinuclear area;
however, in some tumors, it was expressed
weakly in the cytoplasm of the cancer cells (Fig.
2A, B, and C). Even though there was some
variability, the staining was more homogeneous
within the tumors than that in normal appearing
bronchial epithelium. Seventeen out of 39 (43.6%)
stage I NSCLC specimens showed negative or
trace expression of RAR-β. One of the conspicu-
ous findings in this study was that RAR-β was
more frequently expressed in the adenocarci-
nomas (72.7%) than in the squamous cell carci-
nomas (31.3%) (p=0.026). However, the RAR-β
expression status was not affected by differentia-
tion of the cancer cells, and no differences were
observed between bronchioloalveolar carcinomas
and other adenocarcinoma subtypes. Table 1 sum-
marizes the associations between the RAR-β

expression status and the clinical/pathological
parameters.

Relationship between RAR-β expression in stage I NSCLC tissues and adjacent normal appearing bronchial epithelium

Because loss of RAR-β expression is considered
one of the biomarkers of bronchial preneoplasia,
and its restoration reflects suppression of bron-
chial neoplasia,[10] the relationship between the
expression status of cancer cells and normal
appearing adjacent bronchial epithelium were
evaluated. The RAR-β expression status of tumor
cells did not reflect that of the normal appearing
adjacent bronchial epithelium and *vice versa*. A
representative case, whose tumor cells and adja-
cent bronchial epithelium differ in their RAR-β
expression status, is presented in Fig. 2A (tumor)
and 1A (corresponding adjacent bronchial epithe-
lium). The normal appearing adjacent bronchial
epithelium of 14 out of 19 slides, whose tumors
expressed RAR-β, expressed no RAR-β. The dis-
crepancy in the RAR-β expression status between
normal appearing adjacent bronchial epithelium
and tumor cells has caused us to question the use
of RAR-β expression loss as a biomarker for
bronchial preneoplasia (Table 2).

### Clinical parameters associated with loss of RAR-β expression

The median follow-up time among those
patients still alive was 53.9 months. Thirteen out
of 39 (33.3%) patients experienced a recurrence,
distant metastasis and/or a secondary primary
tumor, and 3 (7.7%) patients died of cancer-related
events during the follow-up period. There were
no statistical differences between the group whose
adjacent bronchial epithelium express no RAR-β

Table 2. Comparision of RAR-β Expression in the Cancer Tissue and Coresponding Normal Appearing Bronchial Epithelium

		RAR-β expression in tumor tissue		
		No expression (n=14)	Expression (n=19)	p-value
RAR-β expression in normal	No expression	7	14	0.273
appearing adjacent bronchial epithelium	Expression	7	5	
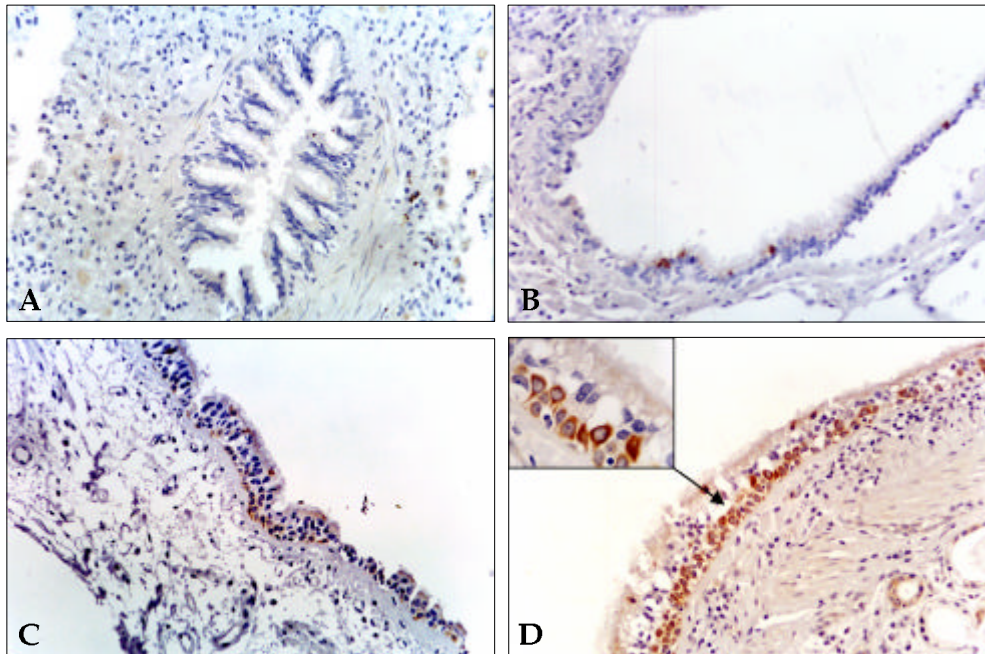
Fig. 1. IHC detection of RAR-β expression in adjacent normal appearing bronchial epithelium. A: There was no RAR-β expressed in the cells comprising the bronchiolar structure (×200). B: Focal, but distinct, RAR-β expression in the bronchial epithelial cells (×200). C: Distinct perinuclear staining of RAR-β in the basal cells of the bronchus (×200). D: Most of the basal cells of the bronchus showed strong perinuclear expression of RAR-β (×200). The inner box shows a higher non-small cell lung cancer magnification of the indicated area (×400).
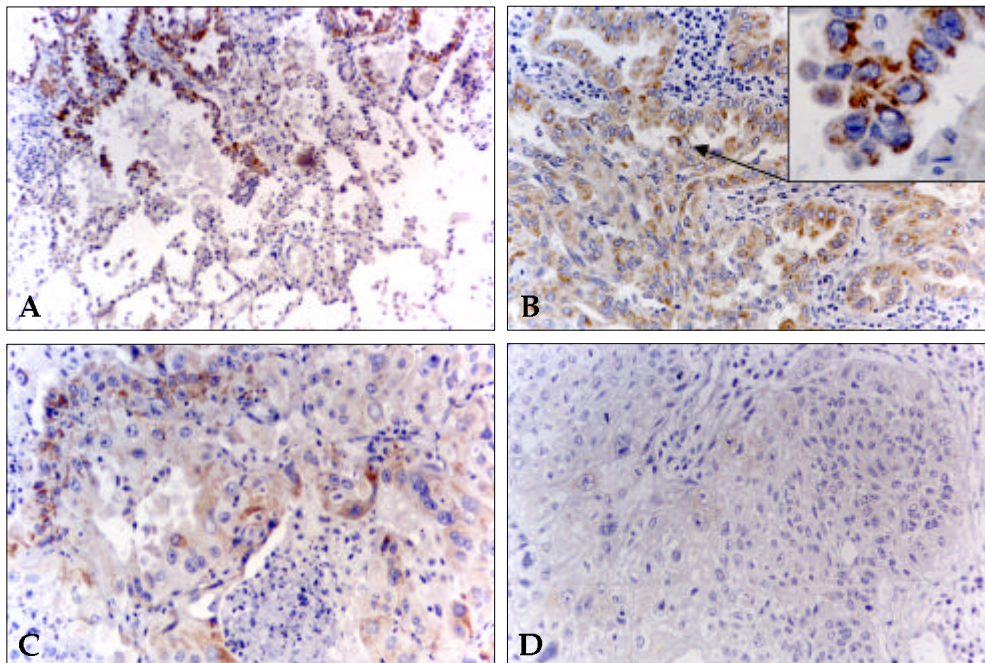
Fig. 2. IHC detection of RAR-β expression in stage I NSCLC tissue. A: RAR-β expression was localized in the perinuclear area of bronchioloalveolar cancer cells, whereas it was not detected in the cells comprising the normal appearing alveolar structure and bronchiolar structure (×200). B: Most of the adenocarcinomas in this study had strong RAR-β expression in the neoplastic cells (×200). The inner box shows a higher magnification of the indicated area (×400). C, D: Squamous cell carcinoma had only weak or no expression of RAR-β (×200).

and those whose adjacent bronchial epithelium expressed RAR-β in terms of gender, age, smoking status (PY), TNM status at the time of initial diagnosis, pathologic diagnosis or histological grade of differentiation of tumor. There were no differences in the disease-free and overall survivals between the groups.

The gender, smoking status, TNM, or histological grade of differentiation of the patients whose tumors expressed RAR-β did not differ statistically from those with negative RAR-β expression. RAR-β was more frequently expressed in the tumors obtained from relatively young patients (mean age (years); 57.3 ± 11.36 vs 63.1 ± 8.97); this difference, however, did not reach statistical significance (p=0.093). In 5 out of 17 (29.4%) patients whose tumors expressed no RAR-β, the cancer recurred during the follow-up time, compared with 8 out of 22 (36.4%) whose tumors did express RAR-β, but this difference did not reach statistical significance. Two out of 17 (11.8%) patients whose tumors expressed no RAR-β died of cancer related diseases during the follow-up time, compared with 1 out of 22 (4.5%) whose tumors expressed RAR-β, but the overall survival between these groups did not differ.

DISCUSSION

Retinoids, a group of natural and synthetic vitamin A analogues, have shown promise as chemopreventive and therapeutic agents, presumably because they modulate the growth, differentiation and apoptosis of normal, premalignant and malignant cells.[12] Their regulatory effect on gene expression is mediated by nuclear retinoid receptors (RARs and RXRs), which are members of the steroid hormone receptor gene superfamily, and function as ligand-dependent, DNA-binding, transcription-enhancing factors.[13,14] A study using lung tissue, obtained from patients who had undergone a lobectomy for lung metastases from primary cancers in other sites of the body (i.e. breast cancers, sarcomas, renal cell carcinoma, melanomas, laryngeal cancer, and colon cancer), as a control, revealed that 88.9% of bronchial epithelium expressed RAR-β. This study, which used *in situ* hybridization, found

that the difference in RAR-β expression between these normal tissues and NSCLC was statistically significant. They also showed that not only distant (91.7%), but also adjacent (80.6%), normal bronchial lung tissue epithelium expressed RAR-β, which was more frequent than in the corresponding NSCLC tissue.[15] In our study, 22 out of 39 (56.4%) stage I NSCLC tissue samples expressed RAR-β, which was similar to other results using ISH.[15,16] However, 12 out of 33 (36.4%) adjacent bronchial epithelium samples expressed RAR-β; this rate was much lower than the findings of Xu (80.6%),[15] although different detection methods were employed. One of the remarkable findings of our study was the 36.4% of coincidence rate between RAR-β expression in the adjacent bronchial epithelium and stage I NSCLC tissues, which has not previously been reported. Chemoprevention, the use of natural or synthetic agents to reverse, prevent or delay carcinogenic progression of invasive cancer,[17] is based on two fundamental concepts: multistep and multifocal carcinogenesis.[18] According to multifocal carcinogenesis, cancer can develop from multiple genetically distinct clones (field carcinogenesis) and the lateral (intraepithelial) spread of genetically related, preinvasive clones.[17] Field carcinogenesis denotes diffuse tissue damage, resulting from carcinogenic exposure (e.g. cigarette smoke), in an entire epithelial field or region, such as the lung, where the wide range of histological changes associated with chronic smoking and cancer include the loss of cilia, cellular atypia, reserve cell hyperplasia, squamous metaplasia and dysplasia, and carcinomas *in situ*.[19] The lower frequency of RAR-β expression in normal appearing adjacent bronchial epithelium, no association between RAR-β expression of NSCLC tissue and normal appearing adjacent bronchial epithelium and the uneven distribution of RAR-β expression in the bronchus, raise doubts regarding the use of RAR-β as a biomarker for chemoprevention, and indicate the urgent need for its verification to this end.

In vivo experiments using ISH have shown no difference in terms of the RAR-β expression between squamous cell carcinomas and adenocarcinomas of the lung.[15] However, the fact that the RAR-β gene is expressed in the majority of

the cell lines derived from lung tumors, except those from lung tumors with epidermoid characteristics,[20] together with the fact that all-trans RA, one of the representative retinoids, is involved in the differentiation and proliferation of type 2 alveolar cells,[7] might support our data where RAR-β was more frequently expressed in adenocarcinomas than in the squamous cell carcinomas of the lung.

Retinoids and RAR-β might play a critical role in the signal pathway that triggers differentiation and limiting of cell division in normal lung tissue. The fact that the expression of RAR-β messenger RNA is decreased or suppressed in a number of tumors, including lung carcinomas,[15,20,21] squamous cell carcinomas of the head and neck[22] and breast carcinomas,[15] suggests that it might be involved in cancer development. There was no apparent relationship between the differentiation status of NSCLC and RAR-β expression, and the loss of expression was not associated with the disease stage when stage I was compared with stages II-IV. This was most likely because this event may have already occurred in premalignant lesions, and was maintained throughout the carcinogenesis process, as has been observed in head and neck carcinogenesis.[22,23] The loss of a specific region on the short arm of chromosome 3 (3p21-24) is a frequent event in nearly all types of human lung cancer. The high frequency of deletions in this region suggests that it contains tumor suppressor genes. Furthermore, a recent report demonstrated that loss of RAR-β expression in neuroblastoma tumor tissue was associated with a poor patient prognosis.[24] However, Khuri et al., using ISH, demonstrated that expression of RAR-β in stage I NSCLC indicates a poor prognosis, and also demonstrated that expression of cyclooxygenase-2 (COX-2) was correlated with RAR-β expression.[16,25] A study using bronchial epithelium from heavy smokers concluded that of tissues that were positive for RAR-β and 69% expressed *h*TERT, whereas only 54% of the RAR-β negative specimens expressed *h*TERT. This suggests that RAR-β expression may be an indicator of increased risk of lung cancer in heavy smokers.[11] These recent publications, from large-scale studies, using data from homogeneous study patients, caused us to question the role of RAR-β as a tumor suppressor. We could not present the prognostic implications of RAR-β expression in this study population because most of the patients remain alive, but prolonged follow-up data might provide significant information on these conflicting hypotheses.

In conclusion, our study has demonstrated that expression of RAR-β in stage I NSCLC differs between adenocarcinomas and squamous cell carcinomas of the lung. In addition, its expression is more frequent in tissues from a relatively young population, suggesting it might be useful for classification of NSCLC. However, the lower rates of RAR-β expression in normal appearing bronchial epithelium compared to the previous studies that employed different methods, together with the inconsistency between the expression status of RAR-β of NSCLC tissue to that of adjacent normal appearing bronchial epithelium, raises doubts regarding the use of RAR-β as a biomarker in chemoprevention. More comprehensive studies involving the mechanisms inducing RAR-β expression in bronchial epithelium, premalignant and NSCLC cells are necessary to define the role of RAR-β in lung carcinogenesis.

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