Global Analysis of Gene Expression in Invasion by a Lung Cancer Model


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INTRODUCTION

Metastasis is a complicated multistep process that involves interactions between cancer cells and their surrounding microenvironments. Previously, we have established a series of lung adenocarcinoma cell lines with varying degrees of invasiveness. Tracheal graft assay confirmed that cell lines with higher in vitro invasiveness had greater in vivo invasive potential. In this study, we used these model cell lines to identify invasion-associated genes using cDNA microarray with colorimetric detection. A more invasive subtype, CL 1-5 F 4, derived from metastatic lung tumor of severe combined immunodeficient mice inoculated with CL 1-5 cells, was combined with CL 1-0, CL 1-1, and CL 1-5 in cDNA microarray screening, cDNA microarray membranes, each containing 9600 nonredundant expressed sequence tag clones, were used to identify differentially expressed genes in these cell lines. For statistical analysis, self-organizing map algorithm was performed to identify the expression patterns. Positive correlation between gene expression levels and cell line invasiveness was found in 2.9% of the 9600 putative genes. On the other hand, negative correlation was found in 3.3% of the genes. The trends of expression of some of the genes were also confirmed by Northern hybridization and flow cytometry. Our data demonstrated that genes related to cell adhesion, motility, angiogenesis, signal transduction, and some other expressed genes varied with varying degrees of invasiveness. Tracheal graft assay confirmed that cell lines with higher in vitro invasiveness had greater in vivo invasive potential. Therefore, instead of using the traditional paired comparisons, we used cDNA microarray in a series of human lung adenocarcinoma cell lines in this report. The human lung adenocarcinoma cell lines of varying invasion abilities and metastatic potentials were established previously (14). The cDNA microarray method, a powerful tool for massively parallel analysis of gene expression, has been applied in various biological studies for identifying differentially expressed genes (12,15–18). By using the cDNA microarray with colorimetric detection system (15, 16) and arrays of 9600 features, we were able to identify metastasis-associated genes on a genome-wide scale in model lung cancer cell lines.

MATERIALS AND METHODS

Cell Lines. Human lung adenocarcinoma cell lines of different invasive and metastatic capacities (CL 1-0 and its sublines, CL 1-1 and CL 1-5; Ref. 14) were grown in RPMI 1640 with 10% fetal bovine serum at 37°C, 20% O2, and 5% CO2.

In Vitro Invasion Assay. CL 1-5 cells were injected into the tail veins of SCID mice to obtain a more invasive cell line than the CL 1 series published previously (14). A highly metastatic cell line was isolated and cloned from the cancer lesion formed in the lung of mice. After four repeated in vivo selections, the cell line was designated as CL 1-5 F 4 and incorporated into the panel of cell lines for microarray analysis.

Invasiveness of the CL 1 series of cell lines was examined by using MICS. In the MICS system, a polycarbonate membrane containing 10 μm pores (Nucleopore Corp., Pleasanton, CA) was coated with a mixture of laminin (50 μg/ml; Sigma Chemical Co., St. Louis, MO), type IV collagen (50 μg/ml; Sigma Chemical Co.), and gelatin (2 mg/ml; Bio-Rad, Hercules, CA) in 10 mM glacial acetic acid solution. The membrane was placed between upper and lower well plates of a MICS chamber. CL 1 cell line series were then resuspended in RPMI 1640 containing 10% NuSerum and seeded into the upper wells of the chamber (5×104 cells/well). After incubating for 24 h at 37°C, cells that invaded through the coated membrane were removed from the lower wells with 1 mM EDTA in PBS and dot-blotted onto a polycarbonate membrane with 3-μm pores. After fixation in methanol, blotted cells were stained with Liu stain (Handsel Technologies, Inc., Taipei, Taiwan, Republic

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4 The abbreviations used are: SCID, severe combined immunodeficiency; MICS, membrane invasion culture system; EST, expressed sequence tag; SOM, self-organizing map.
of China), and the cell number in each blot was counted under a microscope. Each experiment was repeated three times.

**Tracheal Graft Invasion Assay.** A tracheal graft invasion assay was carried out to confirm that in vitro-selected lung cancer cell lines with different invasive/metastatic potentials also possess invasive ability in vivo. Rat tracheas were isolated from Sprague Dawley rats weighing ~200 g. The airway epithelial cells of the tracheas were denuded by repetitive freeze-and-thaw procedures for three times at −70°C. The CL 1-0, CL 1-1, and CL 1-5 cells were cultured to subconfluence before they were harvested. Cells (10⁶) from each cell line were then injected into the isolated rat tracheas. The upper and lower ends of the tracheas were tightened with threads and implanted s.c. in SCID mice. Each cell line was sealed in three different tracheal grafts and each SCID mouse was implanted with one graft. The SCID mice were killed 4 weeks later, and the tracheal grafts were taken out for histological examination. The tumor part of the tracheal graft was sliced at 1-mm intervals. At least three sections were examined for the presence of basement membrane invasion. All animal experiments were performed in accordance with the animal guidelines at the Department of Animal Care, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

**Biotinylated Probe Preparation and Microarray Hybridization.** Five micrograms of the mRNAs derived from each lung cancer cell line were labeled with biotin during reverse transcription and described in previous reports (15, 16). The microarrays (which measured 18 mm × 27 mm) carrying 9600 PCR-amplified cDNA fragments were prepared on nylon membranes by an arraying machine built in-house. The 9600 nonredundant EST clones were Integrated Molecular Analysis of Genomes and their Expression (IMAGE) human cDNA clones, each representing a putative gene cluster with an assigned gene name in the UniGene clustering (19). All experiments of hybridization were performed in triplicate individually. The details of probe preparation, hybridization, and color development were also described previously.

The microarray images were digitized by using a drum scanner (ScanView, Foster City, CA). Image analysis and spot quantification were done by the MuCDA program, which was written in-house and is available.5 Other than using the MuCDA program, the microarray images can be processed by commercial image processing programs to convert the true-color images into gray scale images. The gray scale images can then be quantified by any available microarray image analysis programs written for the fluorescence detection method.

**Northern Hybridization.** To confirm the results of gene detection by the cDNA microarray, 16 differentially expressed cDNA clones, including 10 clones of ascending trend and 6 clones of descending trend in metastasis, were selected from the cluster analysis of the array data; and the entire inserts of the clones of ascending trend and 6 clones of descending trend in metastasis, were isolated from Sprague Dawley rats weighing ~200 g. The airway epithelial cells of the tracheas were denuded by repetitive freeze-and-thaw procedures for three times at −70°C. The CL 1-0, CL 1-1, and CL 1-5 cells were cultured to subconfluence before they were harvested. Cells (10⁶) from each cell line were then injected into the isolated rat tracheas. The upper and lower ends of the tracheas were tightened with threads and implanted s.c. in SCID mice. Each cell line was sealed in three different tracheal grafts and each SCID mouse was implanted with one graft. The SCID mice were killed 4 weeks later, and the tracheal grafts were taken out for histological examination. The tumor part of the tracheal graft was sliced at 1-mm intervals. At least three sections were examined for the presence of basement membrane invasion. All animal experiments were performed in accordance with the animal guidelines at the Department of Animal Care, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

**Flow Cytometric Assay.** The adenocarcinoma cell sublines, CL 1-0, CL 1-1, and CL 1-5-F 4, were subjected to indirect immunofluorescence staining for the expression of surface tumor-associated antigen L6, integrin α-3, and integrin α-6 using murine monoclonal antibody against human tumor-associated antigen L6 (American Type Culture Collection, Manassas, VA), integrin α-3 (Chemi-Con, Temecula, CA), and integrin α-6 (BQ16; Acell Co., Bayport, MN), respectively. The detailed procedures were described in a previous report (16). The fluorescence intensity was analyzed by FACStar (Becton Dickinson, Mountain View, CA).

**Statistical Analysis.** We performed cluster analysis to identify invasion-associated genes on the microarrays. Gene expression data obtained from the microarray experiments were processed and normalized using the protocol and program described by Iyer et al. (18). Genes were clustered into groups on the basis of expression profiles by SOM algorithm as described by Tamayo et al. (20). After cluster analysis by the SOM method, genes whose expression profiles correlated either positively or negatively with the invasiveness of the cell lines were identified. Genes whose expression correlated with the invasiveness of cell lines also were grouped into categories by their putative functions on the basis of literature reports. Genes with multiple roles were included in more than one category.

**RESULTS**

Fig. 1 shows the results of the invasiveness measurement of the four human lung adenocarcinoma cell lines, CL 1-0, CL 1-1, CL 1-5 and CL 1-5-F 4. Cells invading through the coated membrane were harvested and counted. The cell counts were: CL 1-0, 202 ± 16; CL 1-1, 1491 ± 202; CL 1-5, 3865 ± 530; and CL 1-5-F 4, 4115 ± 507. Each bar in the graph represented the average of triplicate measurements. The invasiveness of the four cell lines were as expected and followed a trend of: CL 1-5-F 4 ≥ CL 1-5 > CL 1-1 > CL 1-0. In Fig. 2, the invasiveness of the four adenocarcinoma cell lines was confirmed to have equivalent in vitro invasiveness by the tracheal graft invasion assay. Fig. 2A shows the histochemical staining of the control rat trachea without tumor cell injection and without epithelial cells on the basement membrane. Fig. 2B demonstrates rat tracheal graft injected with isolated normal human airway epithelial cells. After 4 weeks, the human airway epithelial cells repopulated on the rat tracheal basement membrane. The repopulated airway epithelial cells revealed pseudostratified columnar epithelium with mucus and ciliary differentiation. Fig. 2C illustrates tracheal graft injected with CL 1-0 cells. Tumor formation was evident (T, location of tumor). However, histochemical staining revealed no invasion of the basement membrane (arrows). Fig. 2D revealed that, when tracheal graft was injected with CL 1-5 cells, invasion of the basement membrane was clearly evident (Fig. 2D; arrows) in addition to tumor formation in rat trachea. We calculated the invasion foci in three sections of the three cell lines. The total invasion foci/graft for CL 1-0, CL 1-1 and CL 1-5 cells were 0.0, 0.7 ± 0.5, and 4.0 ± 2.0, respectively (ANOVA, 𝛼 = 0.05; 𝑃 = 0.0133).

Biotin-labeled probes deriving from mRNAs of cell lines of varying invasiveness were hybridized to microarrays with 9600 putative genes to profile the gene expression patterns. Fig. 3 revealed a collection of cropped microarray images (4 × 4 spots) showing the gene expression patterns for a series of lung adenocarcinoma cell lines. The trend of gene expression level changes could clearly be seen. The upper panels of Fig. 3 showed the cropped microarray images of the calceclin gene, whose expression levels correlated positively with cell line invasive-

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5 Internet address: ftp://genestamp.ibms.sinica.edu.tw/marray/software./

*Fig. 1. In vitro invasion activity of CL1 sublines. Bars, average number of cells invaded through the matrix gel. Cell numbers: CL 1-0, 202 ± 16; CL 1-1, 1491 ± 202; CL 1-5, 3865 ± 530; and CL 1-5-F 4, 4115 ± 507.*
ness. The lower panels of Fig. 3 contained the AXL gene, whose expression levels also had a positive correlation with invasiveness. From these photographs, it was evident that most of genes (spots), except for calceclin and AXL, had similar expression levels in all of the four cell lines.

To identify all possible metastasis-associated genes from the 9600-feature microarray, we performed cluster analysis on the expression profiles of the four lung adenocarcinoma cell lines. Of the 9600 putative genes, 8525 had statistically significant expression values, and their expression profiles were grouped into 100 clusters. To avoid confusion of negative values in expression patterns, the scale value of normalization, from −1 to +1, was shifted to a positive value, from 0 to +2. The upper four panels (1–4) in Fig. 4A contained four clusters of expression profiles that correlated positively with the invasiveness of the cell lines. Each of the four clusters contained expression profiles of 61, 50, 67, and 99 genes, respectively. The lower four panels (5–8) in Fig. 4A included four clusters of expression profiles that correlated negatively with invasiveness, and each cluster contained 110, 68, 71, and 63 genes, respectively. All of the gene expression profiles in Fig. 4A (277 positively correlated genes and 312 negatively correlated genes) were rearranged by hierarchical cluster analysis with the average linkage method (18), and the expression profiles of 61, 50, 67, and 99 genes, respectively. The super upper four panels (1–4) in Fig. 4A contained four clusters of expression profiles that correlated positively with the invasiveness of the cell lines. Each of the four clusters contained expression profiles of 61, 50, 67, and 99 genes, respectively. The lower four panels (5–8) in Fig. 4A included four clusters of expression profiles that correlated negatively with invasiveness, and each cluster contained 110, 68, 71, and 63 genes, respectively. All of the gene expression profiles in Fig. 4A (277 positively correlated genes and 312 negatively correlated genes) were rearranged by hierarchical cluster analysis with the average linkage method (18), and the expression profiles were grouped into 100 clusters. To avoid confusion of negative values in expression patterns, the scale value of normalization, from −1 to +1, was shifted to a positive value, from 0 to +2. The upper four panels (1–4) in Fig. 4A contained four clusters of expression profiles that correlated positively with the invasiveness of the cell lines. Each of the four clusters contained expression profiles of 61, 50, 67, and 99 genes, respectively. The lower four panels (5–8) in Fig. 4A included four clusters of expression profiles that correlated negatively with invasiveness, and each cluster contained 110, 68, 71, and 63 genes, respectively. All of the gene expression profiles in Fig. 4A (277 positively correlated genes and 312 negatively correlated genes) were rearranged by hierarchical cluster analysis with the average linkage method (18), and the expression profiles were grouped into 100 clusters. To avoid confusion of negative values in expression patterns, the scale value of normalization, from −1 to +1, was shifted to a positive value, from 0 to +2.

The genes clustered in Fig. 4A were grouped into seven categories on the basis of their cellular functions (Fig. 4b). These categories included proteases and adhesion molecules such as interstitial collagenase and integrin α-3; cell cycle regulators such as calceclin and AXL; signal transduction molecules such as transforming protein RhoC and transcription factor activator protein-1 (AP-1); and cytokines and motility proteins such as keratin 18 and myosin light polypeptide 2; angiogenesis-related genes such as matrix metalloproteinase-19 and urokinase-type plasminogen activator; and anonymous genes correlating positively and negatively with invasiveness, marked as ESTs. The genes designated by an asterisk were verified by sequencing. Genes with multiple roles were included in more than one category.

To substantiate the results of the microarray studies, Northern blotting analysis was performed. Ten ascending expressions of genes containing five sequence-verified known genes (such as calceclin and AXL) and five anonymous genes (ESTs) whose expressions had a positive correlation were selected. The other six descending expressions of genes containing five sequence-verified known genes (such as proteoglycan I secretory granule and DnaJ-like heat shock protein 40) and an anonymous gene whose expression had a negative correlation with the invasiveness of adenocarcinoma cell lines were also selected to perform Northern blotting. Fig. 5 demonstrated that the results of Northern blotting analysis were consistent with those from the microarray studies (Figs. 3 and 4b). Radio-labeled glyceraldehyde-3-phosphate dehydrogenase and Gβ-like protein were used as internal controls. In Fig. 5A–5E, and 5L–5P, the known and anonymous genes had higher expression levels in the more invasive cell line (CL 1-5-F 4). On the other hand, in Fig. 5F–5K, the known and anonymous genes were highly expressed in the less invasive cell line (CL 1-0).

To demonstrate that the protein expression of identified genes was also consistent with microarray analysis, three antibodies (tumor-associated antigen L6, integrin α-3, and integrin α-6) were used to carry out flow cytometric analysis across all four CL1 sublines, respectively. Each experiment was carried out in triplicate. Fig. 6A showed the negative control of four CL1 sublines; the average background of fluorescence was 3.3 ± 0.64 (arbitrary fluorescence intensity). In Fig. 6B, antibody against tumor-associated antigen L6 was used to quantify protein expression level; it was obvious that the peak was shifted from CL 1-0 (18 ± 10.0) to CL 1-5-F 4 (233 ± 36.9), and the differentially expressed ratio of CL 1-5-F 4:CL 1-0 was 12.94. Fig. 6C showed that antibody against integrin α-3 made the peak shift from CL 1-0 (3 ± 0.6) to CL 1-5-F 4 (49 ± 17.3), and the differentially expressed ratio was 16.33. In Fig. 6D, antibody against integrin α-6 made the peak shift from CL 1-0 (14 ± 2.8) to CL 1-5-F 4 (53 ± 21.7), and the differentially expressed ratio was 3.79. These results demonstrate that the flow cytometric analyses of protein were consistent with microarray analysis or Northern blotting analysis.

DISCUSSION

It has been well documented that differential expressions of multiple genes and dynamic interaction between various proteins were involved during the multiple steps of metastasis. In this study, we used cDNA microarray with a colorimetric detection system to identify

![Fig. 2. In vivo assay of invasive ability in rat tracheal graft. A, control rat trachea without tumor-cell injection. There were no epithelial cells on the basement membrane. Arrowhead, the cartilage ring of the trachea. B, rat tracheal graft injected with normal human airway epithelial cells. The repopulation of epithelial cells on denuded trachea is indicated by an arrowhead. C, tracheal graft injected with CL 1-0 cells. A tumor (T) was formed but did not invade the basement membrane (arrowhead). Arrow, the cartilage ring of the trachea. D, tracheal graft injected with CL 1-5 cells. The CL 1-5 cells formed tumor and invaded the basement membrane (arrowhead) to form a small tumor nest in the submucosal region (arrow). Original magnifications: A, ×40; B, ×400; C, ×100; D, ×400. Scale bars in each graph: 500 μm, 50 μm, 200 μm, and 50 μm, respectively.

![Fig. 3. Close-up views of microarray images showing gene expression patterns. The order of invasive/metastatic capacities of the cell lines was: CL 1-5-F 4 ≥ CL 1-5 > CL 1-1 ≥ CL 1-0. Arrowheads, genes [calceclin (upper panel) and AXL (lower panel)], which had higher expression levels in more invasive/metastatic cell lines.](cancerres.aacrjournals.org)
Fig. 4a. Cluster analysis of expression profiles of four human lung adenocarcinoma cell lines. A, SOM clustering. Four clusters of expression profiles with ascending trend (1–4) and descending trend (5–8) were selected from 100 clusters. The number of genes in each cluster is indicated at the corner of each panel, and each cluster was represented by the centroid. B, hierarchical clustering. All of the genes derived from A (277 genes with ascending profile and 312 genes with descending profile) were clustered by the average linkage method, and the cluster image shows the trend of gene expression levels in four cell lines with different invasive/metastatic potentials. Fig. 4b. Grouping of invasion/metastasis-associated genes. The genes clustered in Fig. 4 were grouped into categories on the basis of their cellular roles. A, proteases and adhesion molecules; B, cytoskeleton and motility proteins, C, cell cycle regulators; D, signal transduction molecules; E, angiogenesis; F, anonymous genes that correlate positively with invasiveness; and G, anonymous genes that correlate negatively with invasiveness. *, genes verified by sequencing. Genes with multiple roles were included in more than one category.
invasion/metastasis-associated genes on a genome-wide scale in model lung cancer cell lines.

For this study, we established a series of model cell lines with different invasion capabilities both in vitro and in vivo. The tracheal graft invasion assay confirmed that highly invasive cell lines in vitro had retained their corresponding levels of invasiveness in vivo. Gene expressions of these four human adenocarcinoma cell lines were analyzed by cDNA microarray method with colorimetric detection. This powerful tool was very suitable for multiple-gene analysis because it can simultaneously monitor many genes in an experiment. We identified hundreds of genes that were differentially expressed in these model cell lines. Some of these genes showed strong correlation, either positively or negatively, between their expression levels and the invasiveness of cell lines. Several genes, such as calcyclin and AXL, identified by this approach previously have been confirmed to associate with metastasis (21, 22). These findings illustrated that a series of cell lines with varying invasive capabilities analyzed by a cDNA microarray technique could be a good model system in identifying invasion- or metastasis-associated genes.

SOMs, one of the widely used clustering methods, could organize expression profiles into clusters of patterns. This characteristic may also be useful in a study to identify metastasis-associated genes. In our study, 8525 genes were analyzed and their expression profiles grouped into 100 clusters. Four of the clusters (277 genes) contained genes whose expression correlated positively with invasiveness of cancer cell lines; whereas another four clusters (312 genes) had negative correlation to invasiveness. This indicated that only 2.9% and 3.3% of the genes correlated positively or negatively, respectively, with the invasiveness of cancer cells. Consequently, only a small percentage of genes on a genome-wide scale participated in the cellular process that might result in the phenotypic outcome of invasion and metastasis.

Among the 9600 putative genes, 1875 clones on the current version of this microarray are correctly identified by resequencing. One hundred and ten of 589 genes that correlated positively or negatively with invasiveness are included in the sequencing verification. The remainder of the clones will be resequenced for verification continually. The correct identities of these genes will be posted at our Web site as they are confirmed by resequencing. A more complete and additional analysis of the results of this experiment can also be found at our Web site.

All of the genes clustered in Fig. 4a were grouped into seven categories. Five categories were based on reported cellular functions, and two were anonymous genes that correlated either positively or negatively with invasiveness. Among these categories were proteases and adhesion molecules, cytoskeleton and motility proteins, cell cycle
regulators, signal transduction molecules, and angiogenesis-related molecules. In the category of proteases and adhesion molecules, we found that molecules such as disintegrin-metalloprotease, matrix metalloproteinase-19 protein, interstitial collagenase, protocadherin, integrin α-3, and integrin α-6 were included in this group. Most of these genes have been reported previously to associate with cancer invasion and metastasis (23–26). Our data on integrin α-3 and integrin α-6, derived from either microarray analysis or flow cytometric analysis, was consistent with these previous reports.

Metastasis is associated with the movement of cells. Therefore, genes whose expressions are involved in the cellular cytoskeleton and motility may play an important role in metastasis. A previous study illustrated an association between invasiveness and the expression of keratin-18 in highly metastatic cells (14). Another report demonstrated the interaction between a heavy chain of nonmuscle myosin and Ms1 protein, which has been implicated in the formation of metastatic phenotype via the regulation of cell motility and invasiveness (27).

Among cell cycle regulators, calcyclin is a small calcium-binding protein that regulates the cell cycle and is reported to have a positive correlation with metastatic capacity in highly metastatic human melanoma cell lines (21). The expression of AXL, a receptor tyrosine kinase, is also higher in a metastatic prostate carcinoma cell line as compared with normal prostate tissue (22). This molecule has recently been proven to be a mitogenic factor in human thyroid cells (28).

In the category of signal transduction molecules, the expression of the RHOC gene, a member of the p GTPase family, is found to be associated with cell line invasiveness (29). Recently, Clark et al. (30) also reported that the RHOC gene is associated with pulmonary metastasis in melanoma cell lines (human A375 or mouse B16 serial cell lines). Another signal transduction molecule is transcription factor AP-1, which is reported to cooperate with nuclear factor κB to up-regulate the IL-8 gene and thus contribute to the progression and metastasis of human pancreatic cancer (31).

Angiogenesis is another key step in achieving successful metastasis. Many genes involved in metastasis are, therefore, also associated with blood vessel formation. Thrombospondin-2 is reported to have an inhibitory role in angiogenesis. Colon cancer patients with thrombospondin-2 expression have a significantly lower frequency of liver metastasis (32). Another molecule involved in angiogenesis, urokinase-type plasminogen activator, is an enzyme that degrades proteins in tissue basement membrane and extracellular matrix. It is reported that urokinase-type plasminogen activator is expressed in highly metastatic bladder cell lines and significantly elevated in prostate cancer patients with metastasis (33, 34).

The tumor-associated antigen L6 not grouped into the aforementioned categories was a cell-surface antigen that was highly expressed on several carcinomas, such as lung and breast cancer (35). A recent study revealed that tumor-associated L6 antigen seemed to be a cell-surface antigen that was highly expressed in several carcinomas, such as lung and breast cancer (35). A recent report demonstrated that the expression of L6 antigen complied with invasive ability. We conclude that a series of model cell lines with varying degrees of invasiveness and confirmed in vivo metastatic properties evaluated by the cDNA microarray method constitutes a powerful system to identify invasion- or metastasis-associated genes. This technique may also allow us to explore complex interactions of multiple genes in orchestrating the underlying cellular processes of cancer metastasis. Additional characterization of genes identified in this study is currently in progress.

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