Tissue proteomics for cancer biomarker development - Laser microdissection and 2D-DIGE -

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Novel cancer biomarkers are required to achieve early diagnosis and optimized therapy for individual patients. Cancer is a disease of the genome, and tumor tissues are a rich source of cancer biomarkers as they contain the functional translation of the genome, namely the proteome. Investigation of the tumor tissue proteome allows the identification of proteomic signatures corresponding to clinico-pathological parameters, and individual proteins in such signatures will be good biomarker candidates. Tumor tissues are also a rich source for plasma biomarkers, because proteins released from tumor tissues may be more cancer specific than those from non-tumor cells. Two-dimensional difference gel electrophoresis (2D-DIGE) with novel ultra high sensitive fluorescent dyes (CyDye DIGE Fluor saturation dye) enables the efficient protein expression profiling of laser-microdissected tissue samples. The combined use of laser microdissection allows accurate proteomic profiling of specific cells in tumor tissues. To develop clinical applications using the identified biomarkers, collaboration between research scientists, clinicians and diagnostic companies is essential, particularly in the early phases of the biomarker development projects. The proteomics modalities currently available have the potential to lead to the development of clinical applications, and channeling the wealth of produced information towards concrete and specific clinical purposes is urgent. [BMB reports 2008; 41(9): 626-634]

Biomarkers for better clinical outcome of cancer patients

Cancer is one of the most common causes of death due to disease in the developed countries, including Japan (1). Therapeutic modalities developed based on clinico-pathological observations and accrued molecular insights into cancer biology have markedly improved the clinical outcome, including the survival period, for most malignancies in the last decades (1). However, cancer diversity prevents the optimal use of the developed therapeutic modalities. In patient populations grouped by established clinical staging systems, response to treatment and corresponding survival rates never reach 100%, suggesting that certain populations of cancer patients may lose the opportunity to receive more intensive care while others may receive overtreatment. For instance, although the TNM classification has been used worldwide as a useful tool for planning treatment and estimating the prognosis of patients (2), patients at identical clinical or pathological stages exhibit obviously different survival probability (3). Therefore, we need to further investigate the association between clinico-pathological parameters and cancer-related genes to refine the existing tumor classification so that it corresponds more accurately to the response to treatment. For other instance, a novel inhibitor against epidermal growth factor receptor (EGFR) tyrosine kinase, gefitinib (Iressa; AstraZeneca, Macclesfield, UK), improves non-small cell lung carcinoma (NSCLC) related symptoms and quality of life in some patients who do not respond to previous chemotherapy. However, the response rate to gefitinib remains below 20% of NSCLC patients (4-6) and treatment with gefitinib is associated with serious adverse effects, such as severe acute interstitial pneumonia in 5.4% of patients (7). The association between the presence of EGFR mutations and response to treatment with gefitinib has been extensively studied, and its clinical application is under consideration (8). The efforts to identify tissue biomarkers and apply them to clinical modalities will finally benefit the cancer patients by achieving personalized treatment. Early diagnosis is also a critical requirement for better clinical outcomes of cancer patients. Patients with localized tumors have obviously better prognosis than patients with invasive or metastatic tumors. For instance, although the prognosis for lung cancer patients is generally poor and the overall 5-year survival rate is only 19.9% (1), it approaches 72.1% for patients diagnosed with clinical stage IA NSCLC (3). As NSCLC accounts for almost 80% of lung cancers, a substantial number of lung cancer patients can have curative treatment if diagnosed at an early stage. In order to achieve early diagnosis through screening the general population, non-invasive and cost-effective clinical examinations such as ones using plasma biomarkers are needed. However, the existing plasma biomarkers including CEA, NSE, TPA, CA125, CA19-9, Cyfra and ProGRP, have limited sensitivity and specificity for early diagnosis (9). Plasma biomarkers for early diagnosis will undoubtedly contribute to the improve-

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Received 9 September 2008

Keywords: Biomarker, Cancer, Database, Laser microdissection, Medicine, Personalized, Tissue proteomics, 2D-DIGE

http://bmbreports.org

626 BMB reports
ment of clinical outcome for cancer patients.

**Tissue proteomics for biomarker development for personalized medicine**

Cancer is a disease of the genome. The accumulated genomic aberrations are transcribed to the transcriptome, and translated to the proteome with many modifications, then finally transforming normal cells into fully malignant tumor cells. Genome and transcriptome studies have dramatically furthered our understanding of cancer biology. However, since genomic aberrations affect the malignant behaviors of tumor cells only to the degree that they are translated to the proteome, the study of DNA sequences alone may not substitute the study of the proteome. For instance, although transcriptome studies may be based on the assumption that protein expression levels can to some degree be estimated by measuring the corresponding mRNA levels, genome-wide studies suggested the presence of dramatic discordance between mRNA and protein expression level (10), indicating the need for proteomic studies. Proteins cannot function in the form in which they are translated, and posttranslational modifications controlled by the genome such as phosphorylation and glycosylation are essential for their functional properties. As aberrantly modified proteins are known to regulate cancer phenotypes and thus provide a source of biomarker candidates, it is important to examine the mature form of proteins with posttranslational modifications. However, the status of posttranslational modifications of proteins in tumor cells cannot be fully predicted by studying the corresponding DNA sequence or measuring the amount of mRNA. We know that mutations result in altered enzymatic activities, but the effects of mutations on enzymatic activity cannot be predicted by the status of DNA sequences. The genome and transcriptome cannot predict the localization of the proteins in tissues and cells, their association with other proteins or pattern of release into plasma. Therefore, the study of the proteome can provide unique information that is not available by the other omics studies. Taken together, studies on the proteome will identify the final products of the genome that are closely linked to the malignant behaviors of tumor cells. In this sense, we consider the proteome to be a rich source for biomarker identification and subsequent development.

In a clinical setting, tumor tissues are routinely obtained from cancer patients as surgically resected tissues and biopsy samples. As mentioned above, the survival period after surgery varies even when the patients are diagnosed at the same clinical stage, and prognostic information will be useful to decide whether adjuvant chemotherapy will be needed to reduce the risk of metastasis and local tumor growth. When prognosis is evaluated post surgery, tumor tissues are already recovered as surgical specimens and any additional invasive procedures are not required. Prediction of response to chemotherapy and molecular targeting therapy are also desirable because they offer the opportunity to refine therapeutic strategies. Although perfoming a biopsy is invasive and occasionally causes life threatening side effects, if the prediction accuracy is adequately high, it may be justified (11).

Our previous proteomics studies using two-dimensional difference gel electrophoresis (2D-DIGE) showed that tissue proteomics can reliably provide such biomarkers to predict response to treatment. For instance, through the investigation of the proteomic profile of primary gastrointestinal stromal tumors (GIST), we identified plectin as a novel prognostic biomarker (12). These results were validated through the immunohistochemical examination of plectin expression in 210 cases from a single center, which revealed that the 5-year metastasis-free survival rate was 93.9% and 36.2% for patients with plectin-positive and plectin-negative primary tumors respectively (12). Further immunohistochemical examination of additional cases from different hospitals showed equivalent results confirming the prognostic value of plectin (Kikuta et al, manuscript in preparation). A recent study reported that post-surgical treatment of GIST patients with imatinib, a selective inhibitor against type III tyrosine kinases, had significant inhibitory effects on metastasis (13). By monitoring plectin expression, we may be able to select the patients who may have optimal benefits from adjuvant therapy with imatinib. In liver cancer proteomics, we detected the expression patterns of 23 proteins corresponding to the early intrahepatic recurrence of hepatocellular carcinoma (HCC) after curative surgery (14). Moreover, by focusing on the histological differentiation, we identified APC-binding protein EB1 (EB1) as a novel prognostic biomarker for patients with HCC (15). EB1 is involved in the Wnt pathway, which is aberrantly regulated in HCC (16), and its upregulation has been previously reported in HCC (17) and esophageal cancer (18), however its prognostic utility had not been reported previously. Immunohistochemical examination of EB1 expression will help identify cases with high risk of recurrence, the clinical outcome of which may be improved through subsequent close post-operative follow-up and additional treatment. In lung cancer proteomics, we examined the proteomic profile of NSCLC from patients in which the tumor recurred after curative surgery and were then treated with gefitinib (19). We found that nine unique proteins showed different expression corresponding to the patient’s type of response to treatment with gefitinib. Response to treatment was almost perfectly predictable by measuring the expression levels of these nine proteins in surgical material from newly enrolled patients. In esophageal cancer proteomics, we revealed the protein clusters correlating with histological differentiation and lymph node metastasis (20). We also identified a single biomarker protein the immunohistochemical expression of which can be examined to predict the survival period of esophageal cancer patients (Uemura et al, manuscript submitted). Our sarcoma proteomics study using open biopsy samples taken before treatment resulted in the identification of proteins the expression of which can be examined to predict survival after intense treatment. The prognostic value of the identified proteins was successfully validated immunohistochemically on addi-
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Fig. 1. Outline of tissue proteomics methodology followed for biomarker development for personalized medicine at the National Cancer Center. Based on ideas by medical doctors and pathologists derived from problems seen in practice, the research theme is established. We consider that the development of biomarkers to predict the response to treatment is one of the most urgent research themes. A large number of tumor tissues from cases with well-organized clinico-pathological data is obtained from the National Cancer Center Hospital. Laser microdissection is used to recover specific populations of cells enabling accurate protein expression profiling. The protein expression profiles are generated using 2D-DIGE with high sensitive fluorescent dyes and large format electrophoresis devices. Proteomic signatures that may include biomarker candidates are identified through data-mining in which the acquired proteome and clinico-pathological data are integrated. Employing clinically practical tools such as immunohistochemistry, the prognostic or diagnostic value of biomarker candidates is validated using newly enrolled samples. Finally, the validated biomarkers are subjected to real clinical applications established for personalized medicine.

Fig. 1 illustrates the overview of cancer proteomics system for tissue proteomics in the National Cancer Center (Fig. 1).

Tissue proteomics for plasma biomarker development

Most plasma biomarker studies initially examine plasma samples employing high-end proteomics modalities such as a combination of multi-dimensional liquid chromatography, 2D-DIGE and mass spectrometry (21, 22). As plasma protein contents cannot be fully predicted by tissue proteomics, this is a very straightforward strategy. However, as such strategy has not led to the identification of successful plasma biomarkers to date, we...
may need to develop a novel approach.

First, the sheer diversity of the plasma samples makes it difficult to identify practical plasma biomarkers employing a statistical approach alone, a problem that, for a range of reasons outlined below, cannot be overcome as long as plasma contents are examined directly. Although the specificity of the candidate biomarkers will have to be examined in the real population of people who may have the clinical examination for early screening purposes, it is hard to examine plasma samples from all subtypes of non-cancer-bearing people, because of the limitations imposed by sample availability, adequate sample group representation, and the throughputness of proteomic modalities. Thus we will not know the extent to which the plasma contents vary among non-cancer-bearing people until a nation-wide screening is completed. Indeed, the diversity of the plasma contents of non-cancer-bearing people may exceed that of cancer patients. This is in stark contrast to tissue biomarker studies, in which the molecular variations in a given tissue sample group can be to a degree estimated, for instance, on the basis of past pathological observations. In tumor-vs-tumor comparisons, in particular, the degree of variation between the proteomic profiles of the groups compared can be predicted based on the corresponding clinico-pathological data, and the biomarkers identified using a limited number of samples are then successfully validated on an additional sample set.

Existing practical plasma biomarkers, identified by a range of methods, are often released from tumor tissues. For instance, liver cancer cells produce plasma biomarkers such as alpha-fetoprotein (AFP) (23), protein induced by Vitamin K absence or antagonist II (PIVKA II) (24) and glypican-3 (25). The value of these proteins for early diagnosis has not been demonstrated, probably because they may not be released at the early stages of liver cancer, or not be released into the plasma at levels concordant to their expression levels in the tissues, or not be perfectly specific to tumor cells. However, it is reasonable to assume that specific plasma biomarkers, which may finally be shown to be practical in a clinical setting, may be identified among the proteins specifically generated by tumor tissues.

We believe that the functional investigation of proteins identified by tissue proteomics may lead to the identification of biomarker candidates, or at least provide useful information on which plasma biomarker studies can be planned. Although the functional significance of plasma biomarkers is largely obscure, we believe that their aberrant expression can probably be explained on the basis of their molecular background and known cancer biology. For instance, in a transcriptome study on liver cancer cells, we found 214 genes that had significant correlation with higher AFP expression (26). Certain genes among these are known to play a key role in the progression of HCC and other malignancies such as glypican-3 (27). Our observations were consistent with previous reports that demonstrated that AFP-producing liver cancer has a unique DNA methylation (28), transcriptome (29) and proteome (30) profile, and suggested that the genes associated with AFP, but not AFP itself, may play an important role in the exhibition of malignant behavior traits in liver cancer. We speculate that the existing plasma biomarkers may generally have such functional background, and tissue proteomics may support statistics-based plasma proteomics by revealing the proteins aberrantly expressed in malignant tumor tissues.

Tissue proteomics may have the potential to produce biomarker candidates by comparing the tumor tissues with their normal counterparts. We compared the protein expression profiles of esophageal cancer tissues and their normal counterparts by 2D-DIGE, and found 217 unique proteins with different expression levels between the two groups (20). The listed proteins included known plasma biomarkers such as squamous cell carcinoma antigen (SCC), whose diagnostic value has been extensively studied using plasma from esophageal cancer patients (31). We obtained similar results with other malignancies in which normal counterpart tissues were available. Chignard et al identified calreticulin and PDIA3 as the proteins with the highest expression in liver cancer tissues by 2D-PAGE (32). Based on the observation that the fragment of these proteins is present in tumor tissues, they successfully identified them in the plasma of HCC patients (32). In general, the proteins identified in comparative studies between normal and tumor tissues are likely to include plasma biomarker candidates.

The limitation of tissue proteomics for plasma biomarker development is that it is hard to monitor the expression level of a given protein in plasma samples. A technology that will allow the monitoring of the expression of identified proteins in plasma will make tissue proteomics a powerful tool for plasma biomarker discovery. We consider antibodies to be the most preferable probe to monitor the expression of specific plasma proteins, because, considering the low cost of early diagnosis biomarkers in the Japanese health-care system, the development of novel expensive diagnostic devices may not be acceptable in the hospitals and diagnostics companies. We expect that comprehensive antibody libraries will be a powerful tool to link tissue proteomics and plasma proteomics in the near future (33).

**Laser microdissection in tissue proteomics**

There are a number of issues that are unique to tissue proteomics and need to be considered. Tumor tissues contain various types of non-tumor cells such as normal counterpart cells, vascular endothelial cells, and inflammatory cells. In addition, the vascular structures in the tumor tissues may include plasma, which contains large amounts of plasma proteins. Once such tissues are homogenized together for protein extraction, the obtained proteomic features may reflect three factors: the protein contents of individual cells, the ratio of the different cell populations and the vascular content of the tumor tissues. Tremendous efforts have been made to achieve quantitative
protein expression profiling, and a range of so-called quantitative proteomic technologies is available. However, reproducible results may not be achieved if protein sampling is performed without considering tissue heterogeneity.

A remedy for this problem is laser microdissection (LMD). In LMD, specific populations of cells are recovered under microscopic observations. LMD was first reported in 1996 (34), and applied in many biological studies. Proteins recovered from LMD tissues have been subjected to two-dimensional gel electrophoresis (2D-PAGE) (35-38), as well as surface enhanced laser desorption spectrometry (SELDI) (39, 40) and immuno assay (41-44). Among these techniques, 2D-PAGE appears to be the best tool in terms of quantitativity, comparability, reproducibility, and rate of protein identification.

The limited throughputness is a major obstacle in biomarker studies using LMD with 2D-PAGE. As the cells are recovered under direct microscopic visualization in LMD, single slides may have a limited number of target cells, LMD cannot recover a large number of cells in a short time. As the number of cells for tissue proteomics is determined by the sensitivity of the detection methods used, such as silver staining in 2D-PAGE, the time required for LMD is accordingly fixed. In 2002, Craven et al stated that several hours to days were required for them to recover an adequate number of kidney cells for a single 2D-PAGE gel using LMD, and that the number of protein spots detected was still less than desired (45). Similarly, in 2003, Shekouh et al reported that 50,000 cells were retrieved per sample for pancreatic cancer proteomics using 2D-PAGE, and that 14h and 4h of LMD were required for normal and tumor ductal cells, respectively (46).

In our LMD protocol, approximately 3,000 tumor cells per sample are routinely used to generate a single 2D image. Although it may be possible to run 2D-DIGE with a smaller number of cells (50), we do not reduce the number of cells used to maintain a robust, reproducible protocol. In practice, the tissue area subjected to LMD can be measured using the LMD machine (mmi CellCut, Molecular Machines & Industries, Glattbrug, Switzerland) and we use the cells recovered from an area of 1 mm² of a 10 micrometer thick section for a single 2D-DIGE gel. When the proteins extracted from this tissue area are then separated using a large format 2D apparatus, approximately 5,000 protein spots are routinely generated in a reproducible way (54). We consider the damage caused to the proteins of the target cell populations by the laser to be negligible, and in any case under the detection threshold. Indeed, when 2D-DIGE data from independently prepared LMD tissues was compared by scatter plot analysis, the intensity of most protein spots consistently scattered within a 2 fold difference range.

Protein identification cannot be achieved using proteins extracted from LMD tissue samples, because only several micrograms of proteins are recovered (48). Instead, the 2D image of the analytical gel is matched to that of the preparative gel which contains 100 micrograms of proteins extracted from homogenized tissues. The protein spots are recovered from the preparative gels, and the peptides are extracted from the recovered protein spots. We have optimized the protocol of peptide extraction from the gels for protein identification (54).

In 2001, we obtained a novel ultra high sensitive fluorescent dye for 2D DIGE from the R&D team in Amersham Biosciences. As the novel dye was almost 100 times more sensitive than conventional silver staining, we thought that the dye would improve the throughputness of the LMD application. We successfully demonstrated that the novel dye was applicable to LMD tissues, reported the results to Amersham Biosciences, and published the application in 2003 (48). The dye is now commercially available from GE Healthcare Biosciences with the name of CyDye DIGE Fluor saturation dye. The application of CyDye DIGE Fluor saturation dye to LMD was then employed for lung cancer tissues (49), pancreatic cancer tissues (50), gastric cancer tissues (51), esophageal cancer tissues (20), neurons (52), kidney tissues (53), and liver cancer tissues (15). We have published a more detailed description of our protocols in one of our recent publications (54).

**Laser microdissection and 2D-DIGE**

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The effective identification of proteins labeled with CyDye DIGE Fluor saturation dye needs LC-MS/MS instead of MALDI TOF MS. After identifying several hundreds proteins labeled by CyDye DIGE Fluor saturation dye using MALDI TOF MS (17, 48, 49, 55), we have concluded that protein identification is very difficult once proteins are labeled. We speculate that this may be due to the dye for protein labeling possibly preventing peptide ionization. Indeed, the labeled peptides were
not detected by mass spectrometry, even when the mass of the fluorescent dye was taken into account for database searching. In LC-MS/MS, the peptides labeled with CyDye DIGE Fluorescence saturation dye are separated from the unlabeled ones and then subjected to mass spectrometry. In contrast, in MALDI TOF MS, the labeled proteins are mixed with the unlabeled ones, and may inhibit the ionization of unlabeled peptides. It is worth noting that we have used several MALDI TOF MS machines from Applied Biosystems, Shimazu, and Bruker, and obtained the same results.

LMD is still a labor-intensive experiment even with CyDye DIGE Fluorescence saturation dye, depending on the type of tissues dissected. For instance, several minutes are enough to recover an adequate number of cells from HCC tissues, while LMD of esophageal squamous cell carcinoma (ESCC) requires more time; for instance, in a recent study of esophageal cancer proteomics, it took us almost one month to recover enough cells from 145 ESCC samples by LMD (Uemura et al., manuscript in preparation). In our experience, the tissues requiring the most labor intensive LMD sessions are adenocarcinomas such as pancreatic cancer, lung adenocarcinoma, and cholangiocellular carcinoma, as well as the epithelial type of malignant pleural mesothelioma. Therefore, both the tissue type and sample number should be considered when we plan a schedule for LMD.

LMD does not only recover pure tumor cells. We cannot avoid including extracellular proteins, a small number of cells surrounding the tumor cells and plasma proteins present between the tumor cells. Therefore, immunohistochemical studies may be needed to examine the localization of the identified proteins, otherwise the results obtained from tissue proteomics may be misleading or wrongly interpreted.

LMD requires fresh frozen tissues. Frozen tissues are extensively dehydrated after long storage at -80°C degrees or in a liquid or vapor nitrogen tank. In dehydrated tissues, the microstructures are damaged to such an extent that histopathological observations are difficult to achieve. In our experience, frozen adenocarcinoma tissues stored for several years can not be used for LMD even when embedded in OCT compounds. However, because proteins are not degraded after long storage at extremely low temperatures, we extracted the proteins by homogenizing the frozen tissues, and subjected them to proteomic analysis.

LMD requires the operator to receive basic training or have a working knowledge of the relevant histopathology. In our laboratory, the scientists that join the proteomics group are young medical doctors with more than five years working experience in hospitals. Before joining our research project, they have a training course in pathology for at least three months at the National Cancer Center Hospital. They master the basics of pathological diagnosis for specific organs during the three months training period, and start cancer proteomics with the support and mentoring of pathologists.

2D-DIGE system for tissue proteomics

The electrophoresis device is a critical tool in 2D-PAGE experiments. We found that the larger the gel size the more protein spots were observed. The commercially available largest electrophoresis device is EttanDalt ITI (GE Healthcare Biosciences), which has a separation area of 26 × 20 cm. With this device, even when using a gradient gel, certain areas are so crowded with protein spots that many protein spots overlap. In those areas, the reproducibility was diminished because the separation of protein spots differed from time to time. By increasing the gel size and improving the separation performance of 2D-PAGE, this problem can be solved. However, such large format 2D devices have been well recognized for many years, and several research groups have employed large format 2D systems (56-58).

In 2D-DIGE, because all proteins are labeled with fluorescent dye before gel-electrophoresis, the gel images can be obtained by simple laser scanning. As the gels are sandwiched between low fluorescent glass plates, and directly scanned using a laser scanner, the fragility of the large gels does not limit the spot detection. In 2D-DIGE, we can run 2D gels as large as the scan area of the laser scanner with ease. With this notion, we developed a large format 2D device (Bio-craft, Tokyo, Japan), the size of which was adjusted to the scan area of the largest laser scanner (Typhoon, GE Healthcare Biosciences). Using this device, approximately 5,000 protein spots can be observed in a reproducible way. The automated spot recovering machine for this gel size is available from AsOne (Osaka, Japan).

Future perspective of tissue proteomics for biomarker study

We strongly believe that tissue proteomics is a very productive approach to identify biomarker candidates to predict response to treatment, based on our experience of examining more than 1,000 tumor tissues. Although it will take us some time to have a technology capable of uncovering the entire part of proteome, we can identify biomarker candidates by using presently available proteomics methods. As the potential of tissue proteomics is almost established and many fruitful results are being produced, the urgent challenge of tissue proteomics is the successful transfer of the identified biomarker candidates to real clinical applications.

Ultimately, the final goal of biomarker studies is the benefit of patients, and biomarker studies may require unique factors that may not be needed in basic research. For instance, having a medical background so as to understand the clinico-pathological data is essential to make a best use of the tissue samples. Without detailed clinico-pathological information and a strategy to link them to the experimental data, the value
of using tissue samples is diminished. Current clinical problems should be the first priority when determining the research theme, and for this reason it is critical to include pathologists and medical doctors in the biomarker projects, as it would otherwise be difficult to develop biomarkers that will eventually be used in the hospitals. The modalities used are another major concern regarding the clinical application of biomarkers; the examination of biomarkers will have to be performed using cost-effective and conventional methods such as ELISA and immunohistochemistry. As basic researchers alone may not be able to take into account all aspects of such concerns, intimate collaboration between the basic scientists, the medical staff and the diagnostic companies is essential at the early phase of biomarker studies, and may be one of the most critical points in successfully developing a biomarker.

Acknowledgements
This work was supported by a grant from the Ministry of Health, Labor and Welfare and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan. We appreciate Dr. Hisao Asamura (National Cancer Center Hospital) for an excellent photograph of surgical operation.

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