The Use of Transcriptional Profiling to Improve Personalized Diagnosis and Management of Cutaneous T-cell Lymphoma (CTCL)

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Abstract

\textbf{Purpose:} Although many patients with mycosis fungoides presenting with stage I disease enjoy an indolent disease course and normal life expectancy, about 15\% to 20\% of them progress to higher stages and most ultimately succumb to their disease. Currently, it is not possible to predict which patients will progress and which patients will have a stable disease. Previously, we conducted microarray analyses with RT-PCR validation of gene expression in biopsy specimens from 60 patients with stage I–IV cutaneous T-cell lymphoma (CTCL), identified three distinct clusters based upon transcription profile, and correlated our molecular findings with 6 years of clinical follow-up.

\textbf{Experimental Design:} We test by RT-PCR within our prediction model the expression of about 240 genes that were previously reported to play an important role in CTCL carcinogenesis. We further extend the clinical follow-up of our patients to 11 years.

\textbf{Results:} Our findings demonstrate that 52 of the about 240 genes can be classified into cluster 1–3 expression patterns and such expression is consistent with their suggested biologic roles. Moreover, we determined that 17 genes (CCL18, CCL26, FYB, T3JAM, MMP12, LEF1, LCK, ITK, GNYL, IL2RA, IL26, IL22, CCR4, GTSF1, SYCP1, STAT5A, and TOX) are able to both identify patients who are at risk of progression and also distinguish mycosis fungoides/Sézary syndrome from benign mimickers.

\textbf{Conclusions:} This study, combined with other gene expression analyses, prepares the foundation for the development of personalized molecular approach toward diagnosis and treatment of CTCL. Clin Cancer Res; 1–10. ©2015 AACR.

Introduction

Cutaneous T-cell lymphomas (CTCLs) are rare and sometimes lethal malignancies. CTCLs are a heterogeneous group of non-Hodgkin lymphoproliferative disorders characterized by localization of neoplastic T lymphocytes to the skin (1). Previous epidemiologic studies based on the Surveillance, Epidemiology and End Results (SEER) databases documented that this disease is on the rise in the United States and around the world (2). However, recent findings in the United States suggest that the overall national incidence of this cancer has stabilized at a rate of about 10 cases per million per year in the last decade (3). Different regional variations in CTCL incidence have been reported, where about 14 to 16 cases per million individuals per year were diagnosed in San Francisco, California, whereas only 6 to 7 cases per million individuals per year were diagnosed in Iowa during 2000 to 2009 (3). Recent studies also revealed that CTCL may occur in married couples (4) and cluster in families (5). Recent investigations demonstrated geographic clustering of CTCL cases, therefore implying possible existence of an environmental trigger for this malignancy (6), although this remains to be confirmed.

Mycosis fungoides and Sézary syndrome together represent the most common forms of CTCL and account for >50\% of all CTCL cases (1). In Caucasians, mycosis fungoides/Sézary syndrome affect individuals older than 55 years, whereas in African Americans, Hispanics, and Arabic individuals, this disease can present at a significantly younger age (i.e., 20s and 30s; ref. 7).

In early disease stages that can last for many years, classic mycosis fungoides presents as flat erythematous skin patches and thin plaques with sharply defined borders resembling benign inflammatory dermatoses, whereas at later stages, mycosis fungoides cells can form thicker plaques or tumors over larger areas of skin and may disseminate to lymph nodes and internal organs (1). Early diagnosis of CTCL is often challenging, as this cancer can masquerade clinically as other entities such as chronic eczematous...
Translational Relevance

The majority of mycosis fungoides cases (i.e., ≥70%) present with stage I disease and approximately 80% of these patients experience an indolent clinical course. Unfortunately, it is currently not possible to predict which patients will progress and which ones will remain stable based on the available clinical or pathologic criteria. Furthermore, at early stages, this malignancy often masquerades as chronic eczema, psoriasis, or other benign inflammatory dermatoses, which often delays the definitive diagnosis for months or years. The presented three-cluster molecular signature model highlights novel prognostic markers, many of which are already linked to disease pathogenesis. Moreover, comparison of gene expression between mycosis fungoides/Sézary syndrome and benign inflammatory dermatoses uncovers a panel of 17 markers that are able not only to identify patients, who are at risk of progression, but also distinguish mycosis fungoides from its benign mimickers. This knowledge will aid future molecular diagnosis and prognosis for these cancers.

dermatitis, psoriasis, or fungal infections (8). Even histopathology and current PCR studies for T-cell receptor clonality are sometimes not sufficient for definitive diagnosis (8). This, unfortunately, results in a delay, often by many years, in the diagnosis of this cancer.

In advanced disease, malignant cells can sometimes spread to involve lymph nodes and peripheral blood, leading to the leukemic form of mycosis fungoides. Sézary syndrome is now believed to be a distinct entity, different from leukemic mycosis fungoides, as it often presents de novo without any evidence of preceding mycosis fungoides (8), arises from different cancer-initiating cells (i.e., central memory T cells; ref. 9) and carries a more uniformly poor prognosis (8). This leukemic form of CTCL is characterized by a triad of erythroderma, lymphadenopathy, and detection of malignant T cells with cerebriform nuclei on a peripheral blood smear (1).

Clinical disease stage at the time of diagnosis remains the best predictor of survival and progression for mycosis fungoides. Early stages (i.e., stages IA and IB) often exhibit an indolent disease course, with normal or near normal life expectancy (10–12). In contrast, advanced stages and/or Sézary syndrome are associated with recalcitrant disease and poor 5-year survival rate (1). The majority of patients with mycosis fungoides present with an early stage (i.e., IA or IB) disease (10–12). However, about 15% to 20% of these patients will progress to higher stages and may ultimately succumb to their cancer (10–12). At the same time, whereas many patients with advanced CTCL stages will experience an aggressive course of disease progression, this is highly variable and a small minority of these patients survive for much longer than 5 years. Improving our ability to effectively diagnose mycosis fungoides/Sézary syndrome (i.e., being able to distinguish it molecularly from benign mimickers) and, most importantly, developing molecular tools to identify patients at risk of progression at early disease stages will enable us to personalize our management approach toward diagnosis and treatment of this cancer.

To discover novel prognostic molecular markers and to gain additional insight in disease etiology, we had previously performed a microarray and subsequent RT-PCR analyses of gene expression in biopsy specimens from 60 patients with stage I–IV mycosis fungoides/Sézary syndrome (13, 14). These patients were initially followed for 6 years. The original gene expression analyses revealed three distinct transcription profile clusters (i.e., clusters 1–3), where clusters 1 and 3 contained a mix of stage I–IV disease patients, whereas cluster 2 contained mostly stage I and only a few cases of advanced disease patients (13, 14). All patients with stage IV mycosis fungoides/Sézary syndrome fell into clusters 1 and 3 (13, 14). The described three distinct transcription profile clusters were associated with different clinical courses. Cluster 2 genes corresponded to the best clinical outcome and good response to therapy, whereas clusters 1 and 3 molecular signature patterns were associated with the worst and intermediate clinical outcomes, respectively, and poor response to therapy (13, 14). Because of relatively short initial clinical follow-up period (6 years), these trends did not reach statistical significance at that time (13, 14).

In the current work, we performed a literature review that highlighted about 240 genes whose expression and function is believed to be important in CTCL, pathogenesis diagnosis and/or treatment. We subsequently tested the expression of these genes by RT-PCR in our patient population to identify additional genes that fit into the above-described three-cluster prediction model. A number of genes that fit this model were then selected for further testing and their expression patterns were compared between CTCL lesional skin versus normal skin from healthy volunteers versus lesional skin from patients affected by benign dermatoses that often masquerade as CTCL (e.g., chronic eczema, psoriasis, and pityriasis rubra pilaris, PRP). This was done to identify which prognostic markers may also have a diagnostic value in this cancer.

Materials and Methods

Patients and samples

All patients were enrolled in the IRB-approved study protocol with informed consent in accordance with the Declaration of Helsinki (13, 14). Patients with CTCL were recruited from the Cutaneous Lymphoma Clinic at the Dana Farber Cancer Institute (DFCI)/Brigham and Women’s Hospital (BWH). All tissue samples were obtained and processed as previously described (13). Briefly, 6-mm punch biopsies from involved skin were collected from patients between January 26, 2003 and June 1, 2005. The obtained 6-mm biopsies were immediately snap-frozen in liquid nitrogen. Tissue was powdered in liquid nitrogen (Cryo-Press, Microtec Co.), and total RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using the iScript RT-PCR Kit (Bio-Rad) according to the manufacturer’s instructions.

The historic cohort of patients from Boston (n = 60), which was initially reported in 2007 (13), was at the heart of extensive research that led to multiple publications in the field (13–20). For these patients, 11 years of clinical follow-up data were analyzed in the same way as in previous reports (13–20). The biopsy samples analyzed in this study are the same samples that were analyzed in our previous articles (13–20). The diagnosis and clinical staging were established according to the diagnostic criteria of CTCL (21).

Similarly, volunteers with normal healthy skin (n = 6) and benign inflammatory dermatoses (n = 17) were recruited from the outpatient dermatology clinic of the University of British
Columbia (Vancouver, Canada) with informed consent (17). Full-thickness lesional skin punch biopsies were obtained under local anesthesia as previously described (13, 14, 17).

Quantitative real-time reverse transcription PCR gene expression analysis

While microarray analysis provides an unprecedented capacity for whole-genome expression profiling, it has a number of inherent pitfalls that have been described elsewhere (22–24). Quantitative real-time PCR (RT-PCR) serves as the “gold standard” method for evaluation of gene expression (23), which was the main reason for using this approach in the current study. Before initiating this study, we searched PubMed, Medline, and Web of Science databases using terms “genetic,” “gene expression,” “gene expression profiling” and ‘CTCL,” “Mycosis Fungoides,” or “Sezary Syndrome” to identify studies published in English. On the basis of this search, >400 studies were reviewed and 241 genes of interest were selected. Gene expression was tested via RT-PCR in CTCL patients’ lesional skin, normal skin from healthy volunteers, and lesional skin from patients with benign inflammatory dermatoses as previously described (14, 17, 19). Primer pair sequences for tested genes and control housekeeping genes are listed in Supplementary Table S1. RT-PCR was performed using the obtained cDNA from patients and iScript RT-PCR mix (Bio-Rad) on Bio-Rad iCycler as previously described (14–16). The expression was standardized using genorm method (25) using ACTB, SDHA, YWHAZ, and HMBS housekeeping genes. For every gene analyzed, the highest expression value in our samples was set as 1-fold of expression similarly to the protocol in our previous studies (14, 19).

Statistical analyses

Disease progression and disease-specific survival were analyzed using XLSTAT software (Addinsoft) to obtain Kaplan–Meier curves as previously described (14). P values were calculated using the log-rank test (26). Patient multivariate analysis was performed using the Cox proportional hazards regression method taking into account multiple progression events for each patient.

Results

The three-signature gene expression model identifies novel prognostic markers for CTCL

One of the important criticisms of our previous microarray and RT-PCR gene expression studies (13, 14) was that they did not include numerous genes that were reported in literature by other authors to be important in the pathogenesis, diagnosis, and treatment of CTCL. To address this concern, in the current study, we conducted a literature search and identified about 240 genes (Supplementary Table S1) that were previously reported to play an important role in CTCL. We tested their expression in our cohort of patients for which extensive clinical follow-up is available. This analysis demonstrated that a number of previously reported genes can be classified into cluster 1, 2, or 3 expression patterns (as shown in Figs. 1–3) and such expression is congruent with their suggested biologic role in CTCL pathogenesis. Proto-oncogenes, inflammatory cytokines, cell cycle, novel cancer testis genes were expressed in aggressive disease clusters 1 and 3 (Figs. 1 and 3 and Supplementary Table S2). In total, 33 of about 240 genes tested fit into cluster 1 (poor prognosis cluster) expression pattern with partial overlap with cluster 3.

Figure 1.
Expanded RT-PCR analysis of gene expression reveals 33 genes that are expressed in poor and intermediate prognosis cluster 1 and 3 patients but not in favorable prognosis cluster 2 patients.
(intermediate prognosis cluster) patients (Fig. 1), whereas 7 gene were preferentially expressed in cluster 3 patients (Fig. 3). In these clusters, we observed the expression of cell survival and cell-cycle genes CCND2, NFKB1, PLK1, NAI1; putative oncogenes JUNB, TOX, AH11; novel cancer testis genes GTSF1, SYCP1 as well as embryonic stem cell genes TCF3, EVA1, CHD1; genes promoting inflammatory T-cell signaling ITK, LCK, FYB, GNLY, CCL18, CCL26, E-selectin; skin homing chemokine receptor CCR4; cytokines (and their cognate receptors) that were reported to be secreted by the Th17 cells IL26, IL17A, IL17F, IL21, and IL21R; the IL22 cytokine; actin-binding protein PLS3; matrix metalloproteinase MMP12; downstream positive regulator of WNT/β-catenin signaling LEF1; transcription factors STAT5A, MXI, and POU2AF; markers of T-cell activation TFRC, IRF4; and other signaling genes, including T3JAM, FOSL1, SHD1A, SERPINB4 (Figs. 1 and 3 and Supplementary Table S2). As described in detail in Supplementary Table S2, many of these genes were reported to play cancer-promoting roles in CTCL and other cancers. Moreover, several of them (e.g., CCR4, IL2RA, and TOX) are recognized or proposed as therapeutic targets in CTCL.

On the other hand, putative tumor suppressor genes CDKN1C, BCL7A DLEU1, miR-205, and CST6; epidermal differentiation genes LCE2B, LOR; TGF-β signaling gene LTBP4; WNT/β-catenin pathway antagonist WIF1 (WNT inhibitory factor 1) and psoriasis susceptibility gene PSORS1C2 were expressed in a favorable prognosis cluster 2 (Fig. 2). Also, IL18 cytokine, which is known

![Cluster 2 expression in CTCL patients](image1)

![Cluster 3 expression in CTCL patients](image2)
to induce IFNγ response (possibly targeting malignant infiltrating T cells), and its downstream target IL1F7 (also known as IL37) were also upregulated in this cluster of patients (Fig. 2). As discussed in Supplementary Table S2, many of these genes were reported to act as tumor suppressors in CTCL and other cancers and were shown to be downregulated in neoplasia.

We previously demonstrated on the basis of 6 years of clinical follow-up that these three-signature gene expression patterns were associated with different clinical outcomes in patients with CTCL (14). However, at that time, these trends did not reach statistical significance (14). In the current work, we extended the clinical follow-up of our patients until 2014 (11 years of clinical follow-up).

The new extended 11-year clinical analysis of CTCL progression confirms our previous observations and documents that cluster 2 had many fewer number of progression events (i.e., advancement to a higher CTCL stage and/or death) than clusters 1 and 3 (Fig. 4A). Log-rank test of the presented Kaplan–Meier analysis documents that these three clusters are statistically different ($P = 0.005$). Similarly, with respect to survival, cluster 2 patients enjoyed a favorable 11-year survival, whereas clusters 1 and 3 patients experienced an overall poor survival (Fig. 4B). Statistical significance was observed for survival differences between the three clusters ($P = 0.034$). All 60 patients were analyzed in the above-described analyses.

Because each cluster had a large number of stage I disease patients (i.e., 11 of 19 stage I patients in cluster 1, 18 of 20 patients in cluster 2, and 14 of 21 patients in cluster 3), we specifically analyzed the progression of these patients toward more advanced disease (i.e., stage ≥ II) with respect to their genetic clusters. As presented in Fig. 4C, cluster 2 stage I patients had the highest 11-year progression rates. Strikingly, none of the cluster 2 stage I patients have progressed toward advanced disease (i.e., progression rate of 0%) during the period of 11 years. In addition, we conducted Kaplan–Meier comparisons for each individual cluster pair. $P$ values for these analyses are presented in Fig. 4D.

Finally, for this patient cohort, we conducted a multivariate analysis of disease progression based on gender, age, and clinical disease stage at the time of diagnosis. As we expected, the clinical stage at the time of diagnosis was a strong predictor of cancer progression in our patients (Supplementary Table S3). Specifically, stage ≥III patients had an about 12-fold risk of progressing to higher stages and/or dying from their disease, when compared with stage I disease patients. Stage II disease patients had a 4.7-fold risk of progression and/or death. Also, consistent with the trends reported in the literature, a weak association was documented between male sex and disease progression (Supplementary Table S3). On the basis of our
analysis, age alone was not an independent risk factor for disease progression (Supplementary Table S3).

Comparison of gene expression between CTCL, normal skin, and lesional skin from benign inflammatory dermatoses patients

The early stages of mycosis fungoides are often difficult to distinguish clinically from other benign entities, including chronic eczema, psoriasis, and PRP (8). Furthermore, detection of T-cell clonality in itself is also not diagnostic of CTCL, as a number of benign dermatoses (e.g., lichen planus, pityriasis lichenoides, lichen sclerosus, and pigmented purpura) too can have a dominant T-cell clone as measured by diagnostic PCR-based techniques (8). Histologic diagnosis is often difficult, as in early patch disease stages, malignant lymphocytes represent only 5% to 10% of the total inflammatory infiltrate. Even in advanced stages, using all available clinical and laboratory tools, it is often difficult to distinguish patients with advanced erythrodermic mycosis fungoides and Sézary syndrome disease from patients presenting with nonmalignant erythrodermas secondary to psoriasis, PRP, and atopic dermatitis (8). Hence, new genetic markers are urgently needed to distinguish CTCL from various benign inflammatory dermatoses.

To address this, we compared the expression of the above-described candidate genes between CTCL lesional skin (n = 60), skin biopsies from healthy volunteers, and patients with chronic eczema, psoriasis, and PRP. Strikingly, numerous genes that were upregulated in clusters 1 and 3 patients with CTCL (Figs. 1 and 3) were preferentially expressed in CTCL but not in benign skin samples (Fig. 5, Supplementary Table S4). Specifically, this analysis demonstrated that CCL18, CCL26, FIB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL26, IL22, CCR4, GTSF1, SYCP1, STAT5A, and TOX can jointly be used as diagnostic and poor prognostic markers in patients with CTCL. This comparative expression analysis further revealed that select genes (SERPINB13 and BC17A) were preferentially upregulated in benign skin conditions but not in CTCL (Fig. 6A). PSORS1C2 and WIF1 cluster 2 genes were expressed in normal skin and in indolent CTCL cases (Fig. 6B and Supplementary Fig. S1).

Discussion

This study summarizes many years of research and follow-up and describes in detail the findings for the Boston/DFCI/BWH mycosis fungoides/Sézary syndrome cohort of patients. In this work, we have completed a comprehensive gene expression analysis for about 240 genes that were identified in our prior studies and/or were suggested by previous literature reports to play an important role in CTCL lymphomagenesis. Expression of these genes was analyzed in the context of the three signature pattern prediction model that we previously described (13, 14). On the basis of 11 years of clinical follow-up, we document that 52 of these genes are preferentially expressed in various genetic clusters that correlate with different disease outcomes (i.e., overall progression, disease-specific survival, and progression of stage I patients to more advanced stages). We further compare the expression of these genes between CTCL and benign inflammatory dermatoses that often mimic CTCL and identify 22 of these genes that are specific for CTCL and 5 genes that are preferentially expressed in benign dermatoses or in indolent CTCL and benign dermatoses.

These results, combined with other expression profiling and meta-analysis studies (27–29), lay the groundwork for the development of personalized molecular approaches toward diagnosis and management of CTCL in the future. As highlighted in our findings, a panel of 17 genes, CCL18, CCL26, FIB, T3JAM, MMP12, LEF1, LCK, ITK, GNLY, IL2RA, IL26, IL22, CCR4, GTSF1, SYCP1, STAT5A, and TOX, can serve a dual role to diagnose CTCL.
and potentially predict poor clinical disease course. This hypothesis will have to be validated in future prospective studies. As suggested by our Kaplan–Meier analysis, these genes may also prove useful in the prognosis of patients with early-stage mycosis fungoides. For some time, in other diseases, a single diagnostic/prognostic marker (e.g., PSA, CEA, CA-125, LDH, or HER2/neu) has influenced medical decision making. However, new molecular genetic approaches may soon enable us to follow a panel of multiple cancer-related genes in our patients (30). Considering that CTCL is a heterogeneous malignancy, following a panel of markers may prove more reliable than analyzing the expression for a single gene (31).

From the above gene list, it is notable that TOX expression was also independently found by 2 separate laboratories to be a robust diagnostic and prognostic marker for this cancer (17). STAT5A was implicated in carcinogenesis in the early stages of CTCL by activating an oncogenic microRNA miR-155 (32). IL22, a Th22/Th17 cytokine, was proposed to be a dominant cytokine in CTCL tumor microenvironment (33). The chemokine receptor CCR4 has long been reported to be highly expressed in Sézary syndrome and mycosis fungoides (34) and is currently an investigational CTCL therapeutic target of mogamulizumab, a humanized anti-CCR4 antibody (34). IL2RA (IL2Rα) is expressed in up to 50% of mycosis fungoides/Sézary syndrome cases. IL2 diphtheria toxin fusion protein (denileukin diftitox) was designed to target this receptor in patients (35). CCL26 was shown to correlate with the clinical itch burden in patients with CTCL (36), whereas another potent T-cell chemoattractant CCL18 has been consistently shown to be upregulated in mycosis fungoides and

Figure 6. RT-PCR analysis of gene expression identified several genes that are (A) upregulated in normal skin and benign dermatoses, but not in CTCL or (B) upregulated in benign skin samples and in indolent/stable CTCL.
correlate with the types of skin lesions (i.e., patch vs. plaque vs. tumors; ref. 37). As evident from this brief overview, molecular markers identified in our study have a direct clinical correlation to disease symptoms and treatment as reported in the literature. By combining this knowledge with similar studies that identified critical molecular diagnostic and prognostic markers (27–29), we hope to improve our ability to effectively manage this cancer.

This study further highlighted ectopic expression of novel cancer testis genes and embryonic genes GTSF1, SYCP1, TCF3, and CHD1 that were expressed in poor prognosis cluster 1 patients, whereas cTAGE1, GTSF1, and THAP11 were preferentially expressed in CTCL, but not in benign skin samples. EVA1 (also known as MPZL2), another poor prognosis marker, is expressed early on in the thymus but then is strongly down-regulated during thymocyte developmental progression (38). Also, previous work suggested that a B-cell–specific gene, B-lymphoid kinase or BLK, is constitutively active in malignant T cells and appears to be a bona fide oncogene that drives malignant T-cell proliferation in vitro and tumor formation in vivo (39). In this study, we confirm the expression of the aforementioned genes in CTCL and demonstrate that another B-cell–specific transcriptional factor POU2AF1 is expressed in poor-prognosis cluster 1 patients. Other important putative CTCL oncogenes confirmed by this study include JUNB, PL53, AHI, and PLK1.

For favorable prognosis genes, this study highlights putative tumor suppressor genes BCL7A, CNDN1C, miR-205, DLEU1, IL18, and WIF1. BCL7A and CNDN1C were previously proposed by our laboratory and others to play important roles in CTCL pathogenesis (15, 16, 40, 41). The miR-205 microRNA was documented to act as a tumor suppressor in melanoma and other cancers and has the ability to discriminate CTCL from other benign entities (42). DLEU1 (deleted in lymphocytic leukemia 1), long noncoding RNA putative tumor suppressor gene, is frequently deleted in B-cell chronic lymphocytic leukemia (B-CLL; ref. 43) and is reported for the gene, is frequently deleted in B-cell chronic lymphocytic leukemia 1), long noncoding RNA putative tumor suppressor gene, is frequently deleted in B-cell chronic lymphocytic leukemia (B-CLL; ref. 43) and is reported for the gene, is frequently deleted in B-cell chronic lymphocytic leukemia (B-CLL; ref. 43). Hence, it is possible that mycosis fungoides lesions superinfected with bacteria/fungus, recalcitrant treated lesions, or lesions that were exposed to UVB may have a higher expression of IL17 as a result. Hence, future studies will need to clarify whether IL17 signaling is important in certain cases of CTCL or an epiphrenomenon spuriously observed in a subset of mycosis fungoides lesions.

In summary, this study combined with other gene expression profiling analyses prepares the groundwork for the development of personalized molecular approach toward diagnosis, prognosis, and treatment of CTCL. In the future, it will be important to optimize a panel of genes and select 10 robust markers to diagnose and prognosticate this malignancy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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