# A comparative study of Long Interspersed Element-1 Protein Immunoreactivity in Cutaneous Malignancies

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# Abstract

Abstract Background Skin cancer is the most common cancer worldwide and commonly classified into malignant melanoma (MM) and Nonmelanoma skin cancers (NMSCs), which mainly include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The extent to which Long Interspersed Element-1 (LINE-1, L1) ORF1p is expressed in cutaneous malignancies remains to be evaluated. The aim of this study was to assess LINE-1 ORF1p immunoreactivity in various skin cancer subtypes. Method The expression level of LINE-1 ORF1p was evaluated in 95 skin cancer specimens comprising 36 (37.9%) BCC, 28 (29.5%) SCC, and 31 (32.6%) melanoma using the tissue microarray (TMA) technique. Then the association between expression of LINE-1 encoded protein and clinicopathological parameters were analyzed. Results We showed that LINE-1 ORF1p expression level was substantially higher in BCC and SCC patients compared with melanoma samples ($p < 0.001$). BCC cases had higher LINE-1staining intensity scores compared with SCC cases ($p = 0.004$). In SCC samples lower level of LINE-1 ORF1p expression was associated with age lower than the mean ($p = 0.041$), while no significant correlation was found between LINE-1 ORF1p expression and other clinicopathological parameters (all $p>0.05$). Conclusion : According to our observation, LINE-1 ORF1p immunoreactivity in various skin tumor subtypes extends previous studies of LINE-1 expression in different cancers. LINE-1ORF1p overexpression in NMSCs compared with MM can be considered with caution as a tumor-specific antigen for NMSCs. Keywords: Skin Neoplasms; Retroelements; LINE-1 ORF1p; Immunohistochemistry; Tissue microarray; Biomarker


# Background

Cutaneous malignancy is one of the most prevalent tumors involving millions of humans around the world and unfortunately on the rise. Skin cancers are generally classified as malignant melanoma (MM) which represents only 4% of skin cancer cases and non-melanoma skin cancers (NMSC). NMSC includes 2 major subtypes of BCC and SCC amongst others [1, 2]. The incidence rate of NMSC is 18-20 times higher than that MM, however, it constitutes a relatively small percentage of skin cancer deaths [3]. BCC and SCC are rarely fatal whereas 65-74 % deaths due to cutaneous cancer are caused by malignant melanoma [4]. The high cure rate is associated with BCC and SCC especially when the lesion is small and diagnosed in early stages [1, 5]. The early-stage melanoma may be hard to detect but is curable, whereas advanced melanoma has a poor prognosis and a few median survival time [6].

Several risk factors including individual fair skin, blond hair/red hair, freckling, age, gender, personal or family histories, exposure to environmental UVR, high levels of arsenic in drinking water, polycyclic aromatic hydrocarbons, smoking, genetic syndromes and taking immunosuppression are known to induce cutaneous malignancies [7, 8]. Skin cancer is a multistep process with the accumulation of mutations can result in genomic instability which is the hallmark feature of most cancers such as melanoma [9]. One of the mechanisms that are associated with genomic instability is activation of Transposable Elements (TEs).

TEs are categorized into two subgroups of DNA transposons and RNA transposons or Retrotransposons [10]. Retrotransposons are able to propagate themselves through the human genome by means of RNA mediators. Long Interspersed Element-1 (LINE-1, L1) accounts for about 17% of the human genome, however, their ability in constructing eukaryotic genome structure is a key factor throughout the evolution [11]. Most of these elements are 5'-truncated and incapable of retrotransposition but intact and full-length L1 elements are still potent and active sequences in the human genome [12]. A full-length L1 element is ~6 kbp in length and divided into 3 parts including 1) a 5' untranslated region (UTR) comprises an internal RNA polymerase II promoter; 2) two open reading frames (ORF1 and ORF2); 3) a 3' UTR which is finished with a variable polyA tail. ORF1 and ORF2 are translated into an RNA-binding protein (40-kDa) that has chaperone activity and a protein with endonuclease and reverse-transcriptase activities (150-kDa), respectively [10]. ORF1p trimers have a prominent role than ORF2p in retrotransposition events such that ORF1p is generated in excess of the ORF2p [13, 14]. The function of these elements is intrinsically silenced in their promoters by epigenetic modification and several trans-acting factors. L1 activation in germ cells is silenced through PIWI-interacting RNAs pathway and in somatic cells the SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 6 (SMARCA6) helicase and p53 are predominant factors for L1 repression [14, 15].

In normal somatic cells, methylation is a powerful mechanism of control over the activation of the retrotransposable elements in order to avoid genomic instability, chromosomal defects and other genomic rearrangements [16]. Global hypomethylation of genomic DNA is a critical feature of human cancers it may be due to the transcriptional inactivation of LINE-1 elements [17]. LINE-1 promoter hypomethylation has been described in various tumors including lung cancer, colorectal cancers, breast cancer, prostate cancer, liver cancer, ovarian cancer, and esophageal cancer [14].

In non-small cell lung cancer L1 promoter hypomethylation is associated with genomic instability and unfavorable prognosis [18, 19] and in colorectal cancer emerges as an early specific marker [20]. Both invasive and in situ lesions of breast cancer have shown incomplete methylation of LINE-1 promoter resulting in reduced overall survival and therapy resistance in younger patients [21, 22]. In a recent study LINE-1 hypomethylation levels have been observed in melanoma tumors thicker >4 mm compared with normal melanocyte primary cell cultures [23]

It has revealed that the production of ORF1p due to the LINE-1 expression in *in vitro* transfected cells is 1,000- to 10,000-fold higher levels compare to ORF2p [24]. More than half of human cancers express LINE-1 ORF1p so it could be considered as a highly specified tumor marker [17]. Up to now, there is no data regarding the expression of LINE-1 ORF1p in various skin cancer subtypes. In order to achieve immunohistochemically expression data of LINE-1 ORF1p, the present study was performed to explore the LINE-1 ORF1p expression levels by tissue microarray (TMA) in a well-defined series of skin cancer specimens comprising BCC, SCC, and melanoma.

# Methods

## Patient characteristics and cancerous samples

A total of 139 formalin-fixed paraffin-embedded (FFPE) from various skin cancer samples were included in this study. These archival tissue samples were collected from patients with primary skin cancer diagnosed in the Razi and Imam Khomeini Hospitals of Tehran University of Medical Sciences, Tehran, Iran. Patients tumors were diagnosed between 2013 and 2016 and medical records were reviewed in order to collect – clinicopathological parameters comprising age, gender, lesion type, tumor size, ulceration, metastasis, tumor-infiltrating lymphocytes (TILs), invasion (PNI), Breslow thickness and Clark level (in melanoma), and histological grade (in SCC). Not only the patient's data have no interference in their diagnosis and treatment but also were completely kept anonymous. All the study procedure authorized by the Research Ethics Committee of Tehran University of Medical Sciences (Ref no: IR.TUMS.REC.1394.1733).

## Construction of TMA

The entire skin cancer TMAs were constructed as described previously [25-28]. All hematoxylin and eosin-stained slides were reviewed and the most representative areas in different parts of the tumor were marked by a well-experienced pathologist (A-K). Tissue Microarray samples of 0.6 mm diameter were punched out from the selected regions of each tumor samples per single block and precisely transferred into a new recipient paraffin block. The TMA blocks were constructed in 3 copies, each containing 1 sample from a different region of the tumor using tissue-arraying equipment (Minicore; ALPHELYS, Plaisir, France). Then 4µm sections were cut from the completed array blocks and transferred to adhesion microscope slides. These glass slides were used for immunohistochemically staining of LINE-1 ORF1p antigenicity. Mean *H-score* value of three cores was calculated as a final score.

## Immunohistochemistry

Briefly, all the TMA sections were deparaffinized at 60 °C for 20 min and dehydrated with two different alcohol in grade. Endogenous peroxides and non-reactive staining were blocked with 3% $H_2O_2$ for 20 min at room temperature. After washing the tissue sections for three times, antigen retrieval was performed by immersing the tissues in citrate buffer (pH 6.0) for 10 min in an autoclave. The tissue sections were incubated with the primary monoclonal antibody (1:500 dilution), Anti-LINE 1 ORF1 (EMD Millipore, Cat. No MABC1152, CA, USA) overnight at 4°C. TMA slides were then incubated with anti-rabbit/antimouse Envision (Dako, Denmark) as a secondary antibody for 30 min. Staining patterns were visualized by exposure to 3, 3´-diaminobenzidine (DAB; Dako) followed by counterstaining with hematoxylin visualize antigen (Dako, Denmark). Finally, the slides were dehydrated in alcohol, cleared in xylene (Dako), and mounted for examination. Normal human skin tissue and malignant colorectal tissue were used as a negative and positive control for LINE-1 ORF1p immunostaining.

## Immunostaining assessment

Immunostaining of LINE-1 ORF1p was independently evaluated by two well-experienced pathologists (AK and AG) who were blinded to the patients' outcome and pathological information. A consensus was achieved for all samples. The intensity of staining was scored by applying a semi-quantitative system, ranging from negative to strong as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was categorized according to the positive tumor cells as follows: Group 1, less than 25% positive cells; Group 2, 25–50% positive cells; Group 3, 51–75% positive cells; and Group 4, more than 75% positive cells. To compare all the available data, we assigned an overall histochemical score (*H-score*) to each case by multiplying the intensity score by the percentage of positive cells, which yielded a range from 0 to 300. In this study, mean *H-score* was chosen to categorize samples as with high or low LINE-1 ORF1p expression.

### Statistical analysis

For comparison of LINE-1 staining scores in various skin cancer subtypes, we used a two-sided Student's t-test for pairwise comparison between groups. Moreover, Pearson's chi-square and Pearson's R tests were used to analyse the significance of association and correlation between LINE-1 ORF1p expression and clinicopathological parameters. P values less than 0.05 were considered statistically significant.

## Results

Following tissue processing and immunohistochemistry staining of skin tumors, samples with missing of one or two cores were excluded from the study. A total of 95 samples were included in this study. Of 95 cases, 28 (29.5%) were SCC, 36 (37.9%) BCC, and 31 (32.6%) MM. The present study comprises 67 and 28 male & female respectively. There was a male predominance in 3 groups and male to female ratio in SCC, BCC, and MM were as follows: SCC (23 males and 5 females: 4.6), BCC (26 males and 10 females: 2.6) MM (18 males and 13 females: 1.38). Mean age of patients in, BCC, SCC, and MM subtypes of skin cancers were calculated as 70.44±10.2, 67.23± 12.6, and 65.1 ± 14.2 years respectively. Seven (19.4 %), 2 (7.1 %), and 6 (19.4) patients of BCC, SCC and MM subtypes of skin cancer had ulceration in pathological reports. In terms of invasive and in situ forms in SCC, 2 (7.14) patients had in situ whereas 12 (42.8%) had invasive form, and for remaining of SCC patients it was not available. Margin involvement was seen in 1 (2.8%), 2 (7.1 %), and 7 (23.3%) patients of BCC, SCC, and MM patients. Tumor-infiltrating lymphocytes as a prognostic factor and perineural invasion (PNI) was found in 1 (3.6%) and 1 (3.6%) patients of SCC and 6 (19.4%) and 3 (9.7%) MM patients, respectively. Tumor size was available for 8 (28.6%) SCC cases with a mean value of 4-mm. Metastasis and local recurrence were available for 12 (38.7%) and 17 (54.8%) of MM patients respectively. Moreover 7 (22.6 %) MM patients had lymphovascular invasion. Melanoma lesions are categorized with Breslow thickness into ≤1 (thin melanoma) and >1 mm (thick melanoma) [29]. Thin melanoma was found in 2 (6.5%) cases and thick melanoma in 8 (25.8%). The Clark levels in melanoma were also divided into 2 groups: group 1 (Clark levels I and II) and group 2 (Clark levels III through V). Seven (22.6%) melanoma sample were categorized as group 2 and 6 (19.4%) as group 1 (Table 3), for the remaining it was not available. The clinicopathological features of skin cancer subtypes are summarized in Table 1, 2, and 3.

### Analysis of LINE-ORF1p expression and its correlation with clinicopathological features

LINE-1 ORF1p is expressed with variable intensities in the nucleus and cytoplasm of tumor cells. We first tested LINE-1 ORF1p immunoreactivity on skin normal tissue and colorectal tissues (Fig. 1). Mean *H-score* value of three cores was calculated as a final score. Each tumor type was divided into either lower (≤ mean of *H-score*) or higher (> mean of *H-score*) LINE-1 ORF1p expression. The mean *H-score* in BCC and SCC samples were 170.4 and 111.84 respectively, whereas the mean *H-score* in melanoma cases was 46.27. Twenty of 36 BCC samples (55.6%) expressed lower levels of LINE-1 ORF1p, while 16 (44.4%) cases expressed higher levels (Fig. 2). Low expression of LINE-1 ORF1p was seen in 19 (67.9%) of 28 SCC samples, while high expression was found in 9 (32.1%) cases (Fig. 2). Of the 31 melanoma samples, 22 (71%) had low expression and 9 (29%) showed high expression of LINE-1 ORF1p (Fig. 2). We found a highly significant difference between mean *H-score* of LINE-1 ORF1p expression among the three tumor subtypes (all $p<0.01$). Box plot diagram of LINE-1 ORF1p expression has been shown in (Fig. 3). Moreover, the Mann-Whitney *U* test indicated a significant difference in LINE-1 ORF1p expression between BCC and SCC ($p = 0.004$) and melanomas ($p<0.0001$). Also, there was a significant difference between the expression of LINE-1 ORF1p in SCC and melanoma ($p = 0.002$). Whereas, we could not find a significant correlation between studied clinicopathological parameters and LINE-1 ORF1p expression in BCC and melanoma samples (all $p>0.05$) (Table 1 & 3). A trend was evident between LINE-1 ORF1p expression and ulceration ($p = 0.07$). In SCC samples lower level of LINE-1 ORF1p expression was associated with age lower than the mean ($p = 0.041$), while no significant correlation was found between LINE-1 ORF1p expression and other clinicopathological parameters (all $p>0.05$) (Table 2)

## Discussion

In this study we found LINE-1 ORF1p ORF1p immunoreactivity in various skin tumor subtypes extends previous studies of LINE-1 expression in different cancers. As previously reported [30], ORF1p is expressed 200-fold than ORF1p, so tracking ORF1p throughout disease progression can provide valuable insights regarding retrotransposition events or the impact of LINE-1 expression on the genome.

Overall, LINE-1 ORF1p overexpression can be used as a specific hallmark for diagnosing of human malignancies [17]. In the vast majority tumors including approximately 90% of breast, ovarian, pancreatic cancers, more than half of tubular gastrointestinal tract cancers comprising esophageal and colon cancers and also 50% of lung cancers and 40% of prostate tumors, ORF1p can be detected by immunohistochemistry [14, 17]. In the current study, we found significant expression of ORF1p in BCC samples in comparison to SCC and melanoma. ORF1p immunolabeling in BCC samples was higher than those of melanoma samples in contrary to our hypothesis. The reason why the ORF1p expression in NMSC was higher than malignant melanoma requires more attention.

Carcinogenesis in skin tissues is associated with exposure to different environmental factors. Previous reports have shown that some environmental factors like Gamma irradiation and X- rays some environmental triggers like benzo[a]pyrene (B[a]P), organochlorine pesticides, food-borne carcinogens,

extremely low-frequency magnetic fields (ELF-MF) and some heavy metals like mercury, arsenic, aluminum increase L1 retrotransposition events [31-33]. Since the skin is the first line of defense for mentioned factors, so the higher expression of the level of ORF1 due to the exposure to agents is plausible.

Melanoma tumors have higher levels of genomic instability [9] and are associated with hypomethylation of genomic LINE-1 sequences [23, 34]. However, we expected to get more expression level of ORF1p in melanoma, what caused to observe such a decreased level in comparison to other subtypes remains a mystery for us. It is likely that the monoclonal LINE-1 ORF1p antibody referenced in this study that recognizes the sequence corresponding to amino acids 35 to 44 of LINE-1 ORF1p (MENDFDELRE) has not the capability for targeting those sequences in melanoma. It seems that conventional antigen retrieval pathways are not sufficient for retrieving LINE1 ORF1p immunolabelling. In SCC cases, a trend towards increasing correlation of ORF1p expression with age was observed, which support the evidence that age can play a role in developing SCC.

We observed both cytoplasmic and nuclear pattern of LINE-1ORF1p expression in all of the samples, while the cytoplasmic pattern has been predominantly represented in some cancers [17]. In breast cancers, local relapse, as well as distal metastases and poorer overall survival with tumors displaying nuclear L1-ORF1p in contrast to cytoplasmic L1-ORF1p group, have been observed [35]. Distinguishing and quantification between cytoplasmic and nuclear expression can be highlighted in skin tumor subtypes in future studies.

Each individual genome harbors a different complement of 80–100 potentially active L1 elements, this partly explains the variability in somatic insertions of L1 elements within tumors [36]. How many full length - potentially active elements contribute to immunoreactivity for LINE-1 ORF1p and whether observed heterogeneity in these subsets of skin tumor subtypes is related to differences in the inherited complement of active genomic LINE-1 sequences or not remains to be robustly evaluated. Evaluating, such heterogeneity in melanoma cases may pave finding the reason for such differences. Since the tumor microenvironment plays an important role in cutaneous malignancies progression, studying L1 hypomethylation of functional L1 promoters and their genomic sequences in precancerous lesions can offer a genuine reservoir for finding novel targets for both therapeutic purposes and risk assessments in skin cancers. According to our observation, LINE-1 ORF1p immunoreactivity in various skin tumor subtypes extends previous studies of LINE-1 expression in different cancers. LINE-1ORF1p overexpression in NMSCs compared with MM can be considered with caution as a tumor-specific antigen for NMSCs.

# Declarations


### Acknowledgment

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### Authors' contributions

MAZ, EK, ARK, ARG, AK performed the research. AK and ZM designed the research study. ZM, NE, AN-ER, KK contributed essential reagents or tools. AK analyzed the data. MAZ and AK wrote the paper.


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### Availability of data and materials

The data that support the findings of this study are available from the corresponding author, [AK], upon reasonable request.

### Ethics approval and consent to participate

Present study was performed in accordance with the 1975 Declaration of Helsinki. All experiments are approved by the Tehran University of Medical Sciences Research Ethics Committee in Iran (Ref no: IR.TUMS.REC.1394.1570)

### Consent for publication

Not applicable

### Competing interests

The authors declare that there are no conflicts of interest to disclose regarding funding from industrial sources or other disclosures with respect to this manuscript.

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# Tables

Due to technical limitations, tables 1 through 3 are only available as a download in the supplemental files section.

# Figures

**Figure 1**

IHC staining of colorectal, surgical intestinal resection margins in colon cancer and normal skin tissues. A) Positive immunoreactive LINE-1 ORF1p in CRC, B) negative surgical resection margin sample for LINE-1 ORF1p, C and D are representative for normal skin tissues with different magnifications.

**Figure 2**

Immunohistochemical analysis of LINE-1ORF1p expression in different skin cancer subtypes. LINE-1 ORF1p expression in SCC: (A) +3, strong; (B) +2, moderate; (C) +1, weak; (D) 0, no intensity. LINE-1ORF1p expression in BCC: (E) +3, strong; (F) +2, moderate; (G) +1, weak; (H) 0, no intensity. LINE-1ORF1p expression in melanoma: (I) +3, strong; (J) +2, moderate; (K) +1, weak; (L) 0, no intensity.

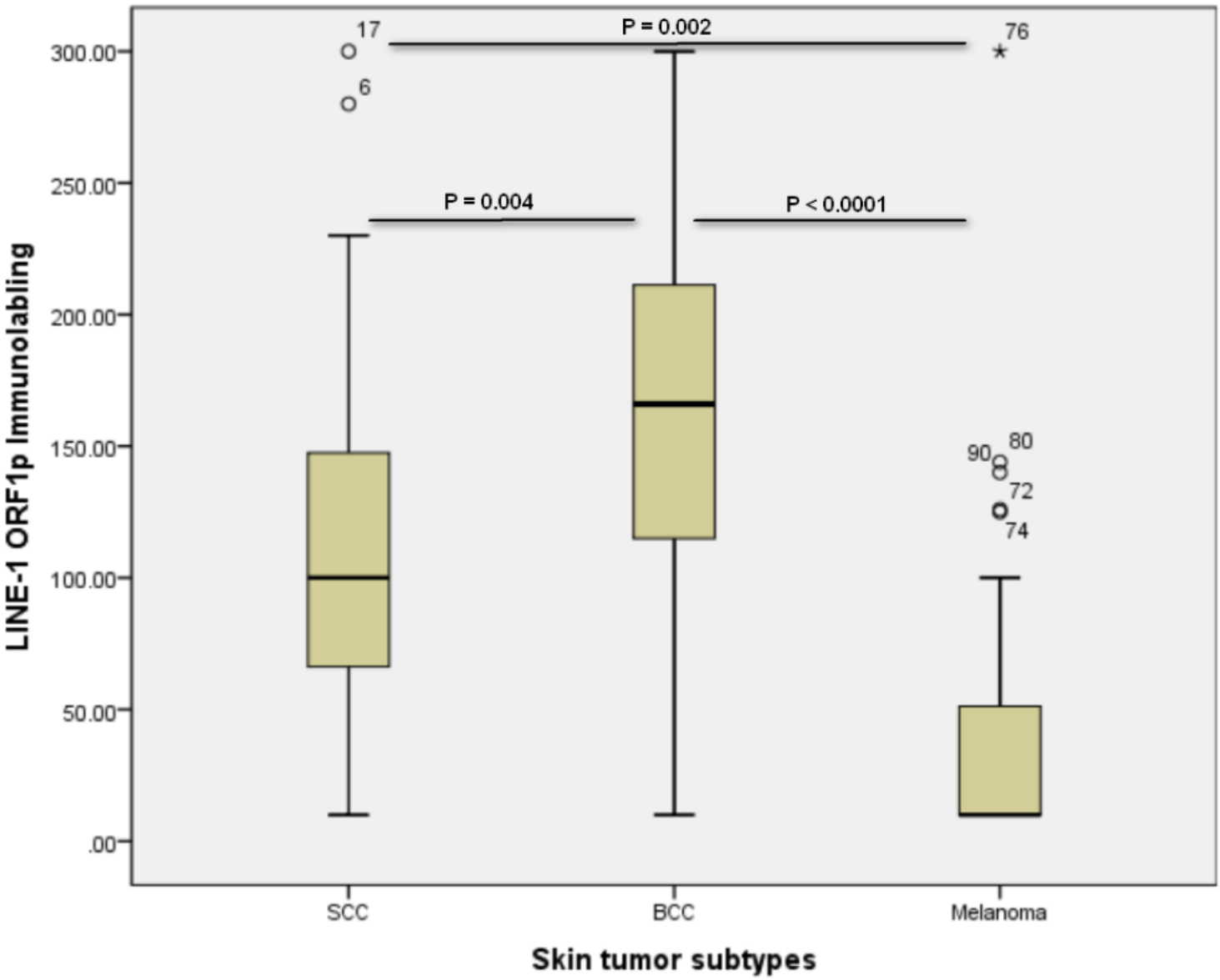


**Figure 3**

Box-plot diagram of LINE-1 ORF1p expression in skin tumor subtypes. In each panel the vertical axis shows the total immunolabeling score (H-score)

## Supplementary Files

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