Surveying lncRNA-lncRNA cooperations reveals dominantly affection on tumor immunity cross cancers

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Abstract

Long non-coding RNAs (lncRNAs) could crosstalk with each other by post-transcriptionally co-regulating genes involved in the same or similar functions; however, the regulatory principles and biological insights in tumor immune are still unclear. Here, we used a multiple-step model to identify lncRNA-lncRNA immune cooperation based on co-regulating functional modules by integrating multi-omics data cross 20 cancer types. Moreover, lncRNA immune cooperative networks (LICNs) were constructed, which are likely to modulate tumour immune microenvironment by regulating immune-related functions. We highlighted conserved and rewired network hubs which could regulate interactions between immune cells and tumor cells by target ligands and activating or inhibitory receptors such as PDCD1, CTLA4 and CD86. Immune cooperative lncRNAs (IC-lncRNAs) playing central roles in many cancers also tend to target known anticancer drug targets. In addition, these IC-lncRNAs tend to be highly expressed in immune cell populations and are significantly correlated with immune cell infiltration. The similar immune mechanisms cross cancers were revealed by the LICNs. Finally, we identified two subtypes of skin cutaneous melanoma with different immune context and prognosis based on IC-lncRNAs. In summary, this study contributes to a comprehensive understanding of the cooperative behaviours of lncRNAs and accelerating discovery of lncRNA-based biomarkers in cancer.

Introduction

Long non-coding RNAs (lncRNAs) are defined as transcripts of more than 200 nucleotides that do not encode proteins, which play crucial roles in diverse biological processes, particularly in diseases such as cancer[1–3]. The function and biological relevance of the lncRNAs in cancer remain enigmatic. Recent studies have suggested that lncRNAs play crucial roles not only in the occurrence and development of cancer with tumor-suppressive and oncogenic activities, but also in cancer immunity, including immune activation and immune cells infiltrating into cancer tissues[4]. For example, BHLHE40-AS1 has been found to support early breast cancer progression by creating an immune-permissive microenvironment [5]. These immune-related lncRNAs could change the tumor immune microenvironment by regulating target genes in cancer, including immune cell infiltration. ImmLnc has been introduced for identifying immune-related lncRNAs and multiple lncRNAs tend to co-regulate the same immune pathways in cancer [6]. Immune checkpoint-associated lncRNAs that were involved in key immune response and immune cell receptor signalling pathways were identified based on coding-genes correlated with lncRNA expression in breast cancer[7]. Together, these studies suggest that lncRNAs may cooperatively regulate the same immune pathways and participate in cancer immunity.

In addition, increasing studies have revealed the extensive cooperative regulation among lncRNAs. Several lncRNAs have been identified to cooperatively dysregulate cancer pathways in multiple tumors[8]. In the functional analysis of lncRNAs, groups of lncRNAs have been identified that are associated with specific cellular processes, suggesting that lncRNA clusters may co-regulate biological processes by cooperatively regulating genes[9–13]. These observations indicate the presence of cooperative lncRNA

regulation, and investigation of the potential functional effects of lncRNA cooperative regulation is interesting. However, it is a challenge to identify lncRNA co-regulation based on experimental methods because of the large number of lncRNA combinations. Context specific lncRNA co-regulation will provide a better approach for inferring the function of lncRNAs in cancer. It is worth to further explore the functions, regulatory roles and biological insights of the lncRNA-lncRNA co-regulation in cancer.

To systematically explore the crosstalk among lncRNAs, cancer-context lncRNA-lncRNA cooperative regulations were identified by integrating multi-omics data based on co-regulating functional modules. We summarized the principles of lncRNA cooperative regulation in immune-related function. LncRNA immune cooperative networks (LICNs) were further constructed. We explored the roles of hub immune cooperative lncRNAs (IC-lncRNAs) in tumorigenesis and interaction between immune cells and tumor cells. We subsequently characterized IC-lncRNAs from expression in immune cell populations and immune cell infiltration. The cancer clusters with similar immune mechanisms were revealed based on the structure of the LICNs and expression of IC-lncRNAs. Finally, new immune subtypes of Skin Cutaneous Melanoma (SKCM) were identified based on six IC-lncRNAs (RP11-71G12, RP11-555F9, RP11-367G6, ITGB2-AS1, AP000233 and AL928768). These analyses and validations of lncRNA-lncRNA cooperative regulation contribute to a comprehensive understanding of the cooperative behaviours of lncRNAs in tumor immune microenvironment.

Results

LncRNAs dominantly co-regulate immune-related functions across cancers.

To explore the function of lncRNAs, we proposed a multiple-step model to identify lncRNA-lncRNA functional cooperation in each cancer type (Fig. 1a). We first identified the cancer context-specific lncRNA-target pairs by integrating multi-omics data. The lncRNA-lncRNA cooperations were further identified based on the cancer-context lncRNA-target regulation across 20 cancer types (Supplementary Table S1)[14]. In each cancer type, one lncRNA pair was considered as cooperation, if and only if they could co-regulate at least a functional module, which was consisted of their shared targets. We identified 6449 lncRNA-lncRNA cooperation pairs among 2145 lncRNAs. The number of cooperative lncRNA accounted for only 16.86% of all the lncRNAs, but these lncRNAs were tightly connected and assembled into an lncRNA-lncRNA cooperative network in pan-cancer (Fig. 1b). Then, we found that approximately 45.13% (968 / 2145) of lncRNAs exhibit cooperation in at least two cancers, on the other hand, 94.73% (6109 / 6449) of lncRNA cooperative interactions are cancer-specific (Supplementary Figure S1). These results indicate that although near half of lncRNAs play cooperative roles in more than one cancer, the cooperative partners of these lncRNAs might be changed in different cancers. Furthermore, by revealing the topological structure with a power-law degree distribution and higher clustering coefficients than randomly linked networks (Supplementary Figure S2a and S2b), the cooperative network of pan-cancer exhibited the scale-free and modular characteristics, indicating that lncRNA-lncRNA cooperative interactions influence each other and effectively exchange regulated information both at a global and at a local scale. Through analysis of the genomic location and expression, we found that the majority of the

cooperative pairs regulate the same function module in trans (Supplementary Figure S3, p = 0.003) and the cooperative lncRNAs tend to have similar expression pattern in cancer types with similar tissue origin (Supplementary Figure S4), such as lower-grade glioma (LGG) and glioblastoma multiforme (GBM).

To further explore the functional characteristics of cooperative lncRNAs, we ranked the functions co-regulated by lncRNAs in descending order according to the number of cooperative lncRNA pairs. We found that all the top functions are related with cancer development and progression, including cell cycle checkpoint, negative regulation of DNA replication and especially many immune-related functions. The proportion of immune-related functions increased with the order and is averagely as high as 58% in top10 to 40 (Fig. 1c). Particularly, the trend of lncRNAs co-regulating immune function is more obvious in lung squamous cell carcinoma (LUSC) and bladder carcinoma (BLCA), and respectively 100% and 90% of top 10 functions are related with immune. As shown in Fig. 1d, 12 (67%) of the top 18 functions are associated with immune, such as T cell activation, lymphocyte activation and interleukin 8 production. Up to 10.7% (696 / 6449) cooperative lncRNA pairs are involved in regulation of T cell activation. In LUSC, each of these immune functions is co-regulated by an average of 208 lncRNA pairs. The lncRNA MAGI2-AS3 could co-regulate cytokine biosynthetic and secretion, interleukin biosynthetic and T cell activation with 9 lncRNAs, and was overexpressed in LUSC (Fig. 1e). It has been proved that MAGI2-AS3 could up-regulate suppressor cytokine signalling 1 and suppress the proliferation of NSCLC cells[15]. The result suggests that co-regulating immune function with other lncRNAs may be another mechanism in cancer development for MAGI2-AS3. Moreover, we found that lncRNAs with more neighbours in the cooperative network tend to co-regulate more immune-related functions (Fig. 1f). These results imply that cooperative lncRNA pairs may contribute to carcinogenesis by regulating immune-related functions in multiple cancers. Our finding may provide a new entry that cooperative lncRNAs have contributions to modify tumor immune microenvironment.

Landscape of lncRNA immune-related co-regulated networks.

To further gain insight into the roles of cooperative lncRNAs in immunity, we extracted all the cooperative lncRNA pairs regulating immune-related functions in each cancer (Supplementary Table S1) and constructed LICNs in 17 cancers respectively based on these pairs(Supplementary Figure S5 and Figure S6). Furthermore, an immune lncRNA-lncRNA cooperative regulatory landscape in pan-cancer was constructed by integrating cancer-specific LICNs (Fig. 2a). This network involves 505 IC-lncRNAs and 1628 IC-lncRNA- IC-lncRNA cooperative interactions across 17 cancer types. Furthermore, we found that the expression of IC-lncRNAs is higher than the other lncRNAs in most of cancers (Supplementary Figure S7a). The IC-lncRNAs pairs also exhibited significantly stronger expression correlation than random in 10 cancers (Supplementary Figure S7b). Thus, we proposed that similar expression patterns might help IC-lncRNAs perform cooperative functions.

Next, we summarized these immune functions into 14 categories and found that these functions are widely distributed, involving multiple levels of immune processes (Fig. 2b). Overall, lymphocyte activation was co-regulated by the largest number of IC-lncRNAs. Moreover, we also found that some function

categories with internal connections share more cooperative IC-lncRNAs, such as lymphocyte activation and T cell activation (Fig. 2c). The trend of the co-regulation for IC-lncRNAs to lymphocyte activation may offer new candidates for cancer immunotherapy. Next, we explored the co-regulation of IC-lncRNAs to immune function categories in each cancer. On average, each cancer type yielded 111 co-regulations among 63 lncRNAs participating in the immune process. The higher number of IClncRNA co-regulations were identified in the cancer types where immune checkpoint-blocking drugs are applicable in clinical (Fig. 2d)[16]. For example, there are 99 IC-lncRNAs and 737 co-regulation pairs in LUSC (Fig. 2e). These IC-lncRNAs accounted for ~ 23.5% (505 / 2145) of all cooperative lncRNAs (Fig. 2d). In addition, the LICN of SKCM contains 110 cooperations among 73 IC-lncRNAs (Fig. 2g). For example, IC-lncRNA LINC00324 which was highly expressed than others co-regulated 10 immune-related functions, such as lymphocyte activation, cytokine biosynthetic and secretion, and interleukin biosynthetic with 49 IC-lncRNAs. A previous study concluded that LINC00324 could regulate the expression of FasL, an apoptosis suppressor concentrated in immune cells and cancer cells, which has been shown to play a vital role in immune evasion[17]. Higher number of IC-lncRNAs regulated the T cell activation and lymphocyte activation across cancer types (Fig. 2d). Particularly, 489 IC-lncRNA pairs are involved in regulation of T cell activation in LUSC (Fig. 2f). Activation of T cells has become an important way to promote antitumor immune response in lung cancer[18]. Moreover, 51 IC-lncRNAs co-regulate T cell differentiation in SKCM (Fig. 2h). The dysregulation of T cell differentiation has been shown in melanoma progression [19]. These results imply that the IC-lncRNAs might play critical roles in tumor immune microenvironment.

Conserved and rewired LICN hubs in tumor immunity.

A few nodes with a large number of neighbours as hubs hold the nodes together in each network. The presence of hubs seems to be a general feature of all biological networks, and these hubs fundamentally could determine the behaviour of networks[20,21]. To investigate the crucial nodes in the pan-cancer LICN, we first identified the top 10% of nodes with the highest connectivity as hub IC-lncRNAs. Then, we assessed the roles of these hub IC-lncRNAs among the different cancer LICNs and grouped these hubs into three categories: common hubs, cancer-specific hubs and other hubs. The common hubs signify the central roles of IC-lncRNAs in more than one cancer; the specific hubs identify IC-lncRNAs with specific central roles in a given cancer. In total, 39.2% (20 / 51) of hubs are common hubs, 39.2% (20 / 51) are specific hubs, and the remaining 21.6% are other hubs. We found that all the hub IC-lncRNAs are extensively involved in immune functions (Fig. 3a). The distribution of three categories of hubs is different across cancers; both LUSC and lung adenocarcinoma (LUAD) contain many common hubs. We further investigated whether three types of hubs have different roles in cancer immune. We found that most of these hubs co-regulated the majority of the immune function categories, such as T cell activation, cytokine biosynthetic and secretion and B cell activation (Fig. 3b). On the other hand, several function categories tend to be specifically co-regulated by different categories of hubs, such as inflammatory response, which are regulated only by the common hubs AC109826, RP11-25K19 and RP11-420G6, and interferon gamma biosynthetic are specifically regulated by the cancer-specific hubs

(Fig. 3b). These results suggested that these hub IC-lncRNAs might play important roles in immune-related functions.

We further explored the contribution of the IC-lncRNAs to cancer development. First, we found that 18 out of 51 hub IC-lncRNAs could participate in cancer hallmarks (Fig. 3c). 9 hub IC-lncRNAs, particularly common hubs, regulate evading immune detection. At the same time, these IC-lncRNAs could regulate other non-immunological hallmarks. For example, other hub IC-lncRNA CTC-241F20 specifically regulated insensitivity to antigrowth signals. The results suggested that IC-lncRNAs may synergistically regulate immune-related functions and other cancer-related processes to involve in the development of cancer. We next found that 8 hub IC-lncRNAs are experimentally validated cancer-related lncRNAs, including 3 common hubs and 5 specific hubs (Fig. 3d). For example, the common hub MIR155HG with 17 partners to co-regulate B cell and T cell activation, cytokine biosynthetic and secretion has been associated with multiple cancer types, including CESC and KIRC. MIR155HG is also a hub IC-lncRNA respectively in CESC and KIRC LICNs. MIR155HG was known as an oncogenic lncRNA and dysregulated by PRDM1 in natural killer/T-cell lymphoma[22]. Then, we further investigated the expression of hub lncRNAs. We found that the average expression level of other hubs is higher than common hubs (Supplementary Figure S8, two-sided t test p = 0.011). These results suggest that IC-lncRNAs may provide a candidate list of cancer-related lncRNAs. Then, we investigated the association between hub IC-lncRNAs and anticancer drugs. We found that 66 out of 84 Food and Drug Administration (FDA) approved anticancer drugs could target hub IC-lncRNA target genes. The common and specific hubs have significantly more anticancer drug targets than other hubs, respectively with an average of 29 and 26 drug targets (Fig. 3e, two-sided t-test, p = 4.39e-05, p = 8.55e-04). For example, some target genes of the common IC-lncRNA RP11-445H22 have been used as anticancer drugs targets, such as CTLA4 as target of radotinib in cutaneous melanoma, EGFR and ERBB2 as target of afatinib in metastatic non-small cell lung cancer. The results suggest that target genes of hub IC-lncRNAs are more likely to be druggable and may be potential targets of anticancer drugs.

Tumor immune microenvironment is very complex involving a range of cell types and molecular mechanisms, especially tumor-immune cell interactions[23]. We surprisingly found that some hub IC-lncRNAs (RP11-445H22, RP11-25K19, and LINC00324) could regulate the genes mediating the direct interaction between tumor cells and immune cells such as, T cells, NK cells, DC cells and B cells (Fig. 3f). The common hub RP11-445H22 has the most immune cooperative partners and could regulate CRTAM, CADM1, CXCR3, CXCL10, PDCD1 and CD274 to mediate tumor - T cell interactions as shown in Fig. 3f, which play a role in promoting tumors and suppressing immunity. Among these receptor and ligand genes, CXCL10 and CXCR3 received strong regulation from RP11-445H22 and the regression coefficient respectively was 0.63 and 0.55 in the above lncRNA target identification model. RP11-445H22 is a KCNK15 and WISP2 antisense RNA and it has been found as an oncogene in lung cancer[24,25]. The regulation to tumor-immune cell interactions for RP11-445H22 may be another mechanism to participate in tumorigenesis. LINC00324 is a specific hub in SKCM, and could be involving in tumor cell-NK cell interactions by regulating TNFSF18, TNFRSF18, CRTAM and CADM1. Furthermore, the three hub IC-

lncRNAs could co-regulate many immune functions with 363, 220 and 50 partners respectively, such as lymphocyte activation, inflammatory response and interleukin biosynthetic (Fig. 3g). The regulation of IC-lncRNAs to these genes mediating interactions can be leveraged to understand the cross-talk between tumors and immune cells and provide a promise to develop novel drugs or therapeutic strategies.

IC-lncRNAs were correlated with immune cell (B cells and T cells) infiltration.

Tumor immune microenvironment is broadly populated with immune cells[26]. Therefore, we reasoned that if these IC-lncRNAs participate in tumor immune microenvironment regulation, then they would be more likely to be highly expressed in immune cells and to be correlated with immune cell infiltration in tumors. Firstly, we found that a significantly higher proportion of IC-lncRNAs (90.7%) was expressed in immune cells by analysing the immune cells RNA-seq datasets (Supplementary methods and Fig. 4a, two-sided fisher's exact test, p = 8.08e-92). In particular, 93.5% of the IC-lncRNAs co-regulating B cell-related functions were expressed in B cells. Moreover, we also found that the IC-lncRNAs co-regulating T cell-related functions were significantly more highly expressed than other lncRNAs in T cells and the B cell-related IC-lncRNAs exhibited significantly higher expression in B cells (Fig. 4b, two-sided wilcoxon test, B cells p = 1.32e-10, T cells p = 2.67e-33). These results suggest that IC-lncRNAs exhibit higher expression in immune cell populations.

We next estimated the associations between expression of cooperative lncRNAs and infiltration levels of six immune cells (B cells, CD4 T cells, CD8 T cells, macrophages, neutrophils, and dendritic cells) in each cancer. We found that a large number of cooperative lncRNAs were correlated with immune cells infiltration in most cancers, such as BRCA, LGG, LUSC, SKCM and THCA (Fig. 4c). In particular, 56.3% and 50.2% of cooperative lncRNAs were correlated with CD8 T cell infiltration in LUSC and in SKCM respectively. The correlations between the expression of IC-lncRNAs co-regulating B cell-related functions and B cell infiltration are significantly higher than others in LUSC (Fig. 4d, two-sided wilcoxon test,p = 1.16e-12). For example, the PCED1B-AS1 co-regulated B cell activation with 8 IC-lncRNAs and the correlation coefficient between the expression and B cell infiltration is as high as 0.69 (Fig. 4e). PCED1B-AS1 could govern aerobic glycolysis, and its overexpression was closely related to larger tumor size and shorter survival time[27]. In addition, the correlations between the expression of IC-lncRNAs co-regulating T cell-related functions and T cell infiltration are significantly higher than others in SKCM, such as RP11-861A13, RP11-25K19, LINC00324 (Fig. 5f, two-sided wilcoxon test, CD4 T cell p = 3.75e-05, CD8 T cell p = 2.22e-03). LINC00324 co-regulating T cell proliferation, activation and differentiation were correlated with CD4 and CD8 T cell infiltration with correlation coefficients 0.42 and 0.45 respectively (Fig. 4g). Indeed, LINC00324 has been found to be overexpressed in cancer and correlated with aggressive cancer progress [28].

We further used fisher's exact test to investigate whether the IC-lncRNAs were likely to be associated with immune cell infiltration. The infiltration levels of CD8 T cells and CD4 T cells were used to divide the patients into two groups respectively (high immune cell infiltration group and low immune cell infiltration group), and it was found that a lot of IC-lncRNAs co-regulating the T cell functions are differently

expressed in majority of cancer types (Fig. 4h, two-sided fisher's exact test). For example, the expressions of AP001055 and CTD-2587H19 which regulate T cell-related functions are significantly high in LUSC patients with high CD4 T cell infiltration (Two-sided t test, AP001055 FDR = 1.32e-05, FC = 2.05; CTD-2587H19 FDR = 1.04e-05, FC = 2.35) (Fig. 4l). In SKCM, RP11-861A13 has significant higher expression in the group with high infiltration of CD8 T cells (Two-sided t test, FDR = 1.29e-07, FC = 2.17). In summary, these results suggest that IC-lncRNAs exhibit higher expression in immune cells and are associated with immune cell infiltration, further validating the roles of the IC-lncRNAs in tumor immune microenvironment.

IC-lncRNA cooperation reveals similar regulation of cancer immune microenvironment.

Several lines of evidence have indicated that sharing molecular features can reveal similar carcinogenic mechanisms between cancer types[29–31]. Here, we hypothesized that if the cancer types exhibit more similar IC-lncRNA cooperation patterns, they are more likely to be with similar immune mechanism. Then, we computed a paired similarity score based on integrating the expression IC-lncRNAs and structure of the LICNs in each cancer (Materials and Methods). We found that some cancers showed greater similarity to each other than to other cancers, such as LUSC, BLCA, CESC, head and neck squamous cell carcinoma (HNSC), BRCA and SKCM (Fig. 5a and Supplementary Results), in which a general immune related trend has emerged[32]. Especially, LUSC, BLCA and CESC were closely clustered together. 41.2% (74/177) IC-lncRNAs were shared by at least two cancers (Supplementary Figure S9). We focused on IC-lncRNAs that were common for the three cancers (Fig. 5b). We found that these IC-lncRNAs were widely involved in co-regulation of immune-related functions, such as leukocyte activation, lymphocyte activation and T cell differentiation. We further discovered that the expression of shared IC-lncRNAs was significantly higher than other lncRNAs in three cancers (Fig. 5c, two-sided t test, all $p < 9.43e-22$). Both lung cancers and bladder cancer have shown relatively effectiveness under immunotherapy using checkpoint blockade[33]. These observations suggest that IC-lncRNA cooperations might operate in cancer types with similar immune mechanism.

Numerous studies elucidate how various components of the immune system control or contribute to cancer progression, thus revealing their prognostic value[34]. We next investigated whether these IC-lncRNAs are associated with survival of cancer patients. We defined risk scores for each patient based on the expression of shared IC-lncRNAs (Materials and Methods). Using the median risk scores as the threshold, cancer samples were classified into two groups with significantly different overall survival rates respectively in all three cancers (Fig. 5d-f). In LUSC, 21 IC-lncRNAs are protective factors which are highly expressed in the low-risk group and other 15 IC-lncRNAs acted as risky factors of which increased expression is associated with a poor survival outcome (Fig. 5g and h). For example, MIR155HG is significantly down-regulated in high-risk group as a protective factor, hazard ratio of which is 0.77 (Two-sided t test, $p = 7.27e-05$). It has been shown that the expression of MIR155HG could predict overall survival in multiple cancers[35]. The approach based on immune lncRNA-lncRNA cooperation could effectively identify prognostic biomarkers. Collectively, these results suggest that the cooperative pattern

based on both the structure of LICNs and the expression of IC-lncRNAs could reveal new cancer clusters from immune view.

LICN contribute to subtype classification of SKCM.

The skin cutaneous melanoma is a malignancy of melanocytes, which accounts for the most number of deaths from skin cancer[36]. Therefore, we investigated to what extent the IC-lncRNAs can be applied to SKCM molecular subtyping. We first identified the top 10% IC-lncRNAs with larger expression fluctuation and further filtered by hubs in SKCM. Finally, 6 IC-lncRNAs (RP11-71G12, RP11-555F9, RP11-367G6, ITGB2-AS1, AP000233 and AL928768) were obtained. These 6 IC-lncRNAs participate in immune functions such as regulation of B cell activation, immune response, lymphocyte activation and immune system process. These IC-lncRNAs were correlated with immune cell infiltration and they were also more highly expressed in B cells (Supplementary Figure S10A and S10B, two-sided wilcoxon tests, B cell $p = 0.0015$). Next, we found that the SKCM patients can be classified into two subtypes based on the expression of the 6 IC-lncRNAs (Fig. 6a). These IC-lncRNAs are highly expressed in group 2 patients (Fig. 6b, two-sided t test, $p = 2.29e\text{-}3$). There are no significant differences in age, gender, and cancer stages observed between the two subtypes. We further compared six immune cell infiltration levels between two subtypes respectively. We found that group2 patients consistently have significantly higher infiltration levels than group1 patients for all immune cells (Fig. 6c, two-sided wilcoxon test, all $p < 1.80e\text{-}09$). Particularly, average infiltration level of CD8 T cells in group2 is 25%, while infiltration level is only 8% in group1. At the same time, the average infiltration level of dendritic cells (DC) of group2 patients was almost double than that in group1. It has been shown that the presence of mature DC within tumors is a positive prognostic factor in melanoma patients and melanoma-associated DCs is also related with immunotherapy[37].

It has been demonstrated that some indicators are useful biomarkers for predicting the immune response, such as immune cytolytic activity (CYT) and major histocompatibility complex (MHC) scores and the immune score[38,39]. We further investigated the distributions of these scores among SKCM patients. We found that group2 patients had significantly higher CYT, MHC and immune scores than group1 (Fig. 6d, two-sided wilcoxon test, all $p < 1.23e\text{-}12$) Treatment with immune checkpoint blockade has transformed the outcome for patients with melanoma, such as programmed cell death protein 1 (PDCD1) inhibitors, CTLA-4 inhibitor[40]. Therefore we compared the expression of immune checkpoint genes between two subgroups. We found that PDCD1, CD274 and CTLA4 are significantly higher expressed in group2 (Fig. 6d, two-sided wilcoxon tests, all $p < 3.25e\text{-}09$). At the same time, the expression of all the 6 IC-lncRNAs are significantly positively correlated with PDCD1 and CD274 (Fig. 6e, pearson correlation, $p < 0.05$). Each IC-lncRNA was more closely related to PDCD2 than CD274 in expression. In particular, the correlation coefficient between IC-lncRNA ITGB2-AS1 and PDCD1 was as high as 0.58 (Fig. 6f). IC-lncRNA ITGB2-AS1 is antisense to ITGB2 and has been shown to be highly expressed and closely related to immunosuppression in AML[41]. ITGB2-AS1 may be as a potential immunotherapeutic target in cancers.

The degree of tumor infiltration by immune cells can predict a patient's clinical outcome in many cancer types[42,43]. Therefore, we explored the prognostic implications of molecular subgroups. We found that patients with group2 had significantly better overall survival than group1 (Fig. 6g, log-rank test p = 0.02). These results suggest that IC-lncRNAs identify different SKCM subtypes (group1 immunosuppressive subtype and group2 immunoactivated subtype) with remarkable immunology diversity (Fig. 6h), which is helpful to improve personalized cancer management.

Discussion

Accumulating evidence suggests that lncRNAs could crosstalk each other by co-regulate biological functions. However, systematic analysis of lncRNA co-regulation is lack especially in cancer. In the current study, cancer-context lncRNA-target pairs were first identified by a multivariate linear model, which factored in variation (noise) in mRNA expression induced by expression of lncRNA, changes in DNA copy number and promoter methylation. Then, lncRNA cooperation was detected via functional modules based on lncRNA-target pairs data. Recently, mounting studies have found that lncRNAs play the roles in tumor immune microenvironment[4]. However, how lncRNAs contribute to tumor immunity was still known a little. In our study, we provided a multiple-step model to identify lncRNA-lncRNA cooperation based on co-regulating functional modules by integrating multi-omics data and found that lncRNAs tended to co-regulate a wide range of immune-related functions, especially T cell activation. The aim of cancer immunotherapy is to promote the activity of T cells within a tumor and establish efficient and durable antitumor immunity[44]. IC-LncRNAs co-regulating T cell activation may provide new views for cancer immunotherapy. T cells can also target tumor cells in various ways, either directly by eliminating tumor cells or indirectly by modulating the tumor immune microenvironment[44]. Particularly, interactions between ligands and activating or inhibitory receptors are crucial for further regulating T cell activation and tolerance[45]. We found that some IC-lncRNAs could target proteins mediating interactions between T cells and tumor cells such as LINC00324, RP11-445H22 and RP11-25K19. RP11-445H22 could positively regulate CTLA4 and PDCD1 both of which are inhibitory receptors of T cells in LUAD. In addition, RP11-25K19 could also positively regulate CXCR3 and CXCL10 which are activating receptors of T cells in SKCM. These IC-lncRNAs may be as cancer immune therapeutic targets.

Melanoma is a highly aggressive form of skin cancer, where it is often difficult to treat with traditional therapies[37]. The long-term prognosis of metastatic melanoma is poor and prognosis biomarkers remain elusive. We identified two immune-related subtypes of SKCM based on IC-lncRNAs. The two subtypes display obvious diversity in the immune context of the tumor microenvironment and the survival time. The SKCM is also amendable to immunotherapy (PDCD1 or CTLA4 antagonist) for various reasons, including extensive immune cell infiltration[46]. We found that the expression of IC-lncRNAs used to typing are significantly correlated with the PDCD1 and CD274. It is suggested that IC-lncRNAs could contribute to response of prognosis and therapy.

In summary, we presented the lncRNA cooperation landscape across human major cancers and showed the importance of immunity. Our study opens new avenues to investigate the functions and mechanisms of lncRNAs in tumor immune microenvironment. Follow-up investigation is warranted to deepen our understanding of lncRNAs cancer immune functions and their application in clinics.

Materials And Methods

Omics data across cancer types.

The paired lncRNA-mRNA expression profiles as well as DNA methylation and copy number data were obtained from the TCGA. The mRNA expression datasets profiled via RNA sequencing (level 3, TPM) were used. LncRNA expression datasets (FPKM) were obtained from The Atlas of Noncoding RNAs in Cancer (TANRIC)[47]. The expression values of mRNAs and lncRNAs were log2 transformed for subsequent analysis. The methylation level for genes was defined as the average beta-values of probes mapping to the corresponding gene promoter. In addition, DNA copy number datasets were downloaded from Firehose. In total, each cancer type had paired lncRNA and mRNA expression profiles as well as DNA methylation and copy number data measured for the same sample, forming a pan-cancer data compendium from 5284 tumor samples for 17039 mRNAs and 12727 lncRNAs (Supplementary Table S1). The clinical information of patients, including the survival status, stage, age and survival time, was also downloaded from the TCGA project (Supplementary Methods).

Identification of lncRNA-lncRNA cooperation across cancer types.

We proposed a multiple-step model to identify lncRNA-lncRNA functional cooperation in each cancer type. First, a multivariate linear regression model was used to identify cancer-context lncRNA-target regulations. Then, the cooperative lncRNA pairs were identified as follows: for each lncRNA pair, we initially identified lncRNA pairs that significantly shared targets; the shared target genes were used to identify candidate functional modules; and then, the candidate module sets were further filtered using two topological features in the protein interaction network. Here, we defined a pair of lncRNAs as cooperative if they significantly co-regulated at least one functional module.

Prediction of cancer-context lncRNA-target regulation.

The multivariable linear regression model was used to assess the association between the expression of lncRNA and mRNA in each cancer type and considered the effect of methylation and DNA copy number on the expression of mRNAs. For each pair of lncRNA and mRNA, the predicted model was defined as follows:

$$\mathrm{y_j} = \beta_0 + \beta_{\mathrm{CN,j}} \times \mathrm{x_{CN,j}} + \beta_{\mathrm{Me,j}} \times \mathrm{x_{Me,j}} + \beta_{\mu,\mathrm{j}} \times \mathrm{x}_{\mu,\mathrm{j}} + \epsilon$$

Where $\mathrm{y_j}$ is the expression of $\mathrm{mRNA_j}$, $\mathrm{x_{CN}}$, $\mathrm{x_{Me}}$, x_μ are CNV and promoter methylation at $\mathrm{mRNA_j}$ and the expression of lncRNA_μ respectively, β_0 is a the intercept and ϵ is a random error. The lncRNA-target

pairs with FDR less than 0.01 were further filtered with Bonferroni-corrected p value less than 0.1.

Identification of functionally cooperative lncRNA pairs.

We used the method proposed in our previous study to identify the functionally lncRNA-lncRNA cooperations[48], of which briefly steps are as follows: first, the lncRNA pairs sharing at least three target genes were identified as candidates. Then, shared target genes of each candidate lncRNAs pair were used to identify functional modules. The shared targets were performed functional enrichment by hypergeometric test across selected GO terms. Thus, at a given significant level, we can achieve enriched GO terms and a subset of shared targets annotated to each of these GO terms as a candidate functional module. Next, two topological features were further used to filter the candidate module in the protein interaction network: (i) the minimum distance from each gene to others in the subset is no larger than 2. (ii) the characteristic path length (CPL) is significantly shorter than random. 1000 random networks were created using the edge-switch method with the software Mfinder (available at http://www.weizmann.ac.il/mcb/UriAlon/). The p value was defined as the fraction of CPL for the same subset that was shorter than that in the real network. Finally, after performing function enrichment and two topological restrictions in the network, a pair of lncRNAs was considered cooperative if they co-regulated at least one functional module.

Correlations between lncRNAs and immune cell infiltration.

The infiltration proportion of B cells, CD4 T cells, CD8 T cells, macrophages, neutrophils, and dendritic cells were predicted by TIMER respectively[49]. Spearman's rank correlation was calculated between the expression of each lncRNAs and infiltration proportion of immune cells. Wilcoxon rank sum test was used to evaluate the difference between IC-lncRNAs co-regulating corresponding immune cell-related functions and other lncRNAs with immune cell infiltration. In addition, we classified the patients into two groups according to the median of infiltration proportion of CD4 T cell and CD8 T cell respectively in each cancer. Differently expressed lncRNAs between two groups were identified using fold-change and T test (FC > 2 or FC < 0.5 and FDR < 0.05). Fisher's exact test was used to evaluate the significant overlap between differently expressed lncRNAs and IC-lncRNAs regulating T cell related functions.

Evaluation of similarity among cancers based on LICNs.

We evaluated the degree of similarity between cancers based on both the structure of immune lncRNA cooperative networks and the expression of immune cooperative lncRNAs. First, we calculated Jaccard coefficient of IC-lncRNAs in LICNs between any two cancers as R1. Then, the Pearson correlation coefficient was calculated between the expressions of IC-lncRNAs in any two cancers was as R2. Lastly, Hierarchical clustering was used to identify cancer clusters with similar immune mechanisms based on the mean values of R1 and R2.

SKCM subtype classification based on IC-lncRNAs.

IC-lncRNAs in SKCM were firstly filtered with expression value > 0 in larger than 50% of samples. Then, we selected the top 10% of IC-lncRNAs according to the variance of expression in descending order and further filtered by hubs. We lastly identify two SKCM subtypes based on these IC-lncRNAs expression by using ConsensusClusterPlus R package [50] (Supplementary Methods).

Survival analysis of cancer-shared IC-lncRNAs.

The univariate cox regression analysis was used to evaluate the association between survival time and the expression of each cancer-shared IC-lncRNA. We assigned a risk score to each patient taking into account both the strength and positive or negative association of each lncRNA with survival. A risk score per sample i, according to a linear combination of the IC-lncRNA expression weighted by the regression coefficients from univariate cox regression analysis, and was defined as follows:

$$\mathrm{Riskscore} = \sum_{\mathrm{j}=1}^{\mathrm{n}} \beta_{\mathrm{j}} \times \exp_{\mathrm{j}}$$

Where n is the number of IC-lncRNAs, β_{j} is the regression coefficients of IC-lncRNA$_{\mathrm{j}}$ and $\exp_{\mathrm{j}}$ is the expression of lncRNA$_{\mathrm{j}}$. Then, patients were divided into high-risk and low-risk groups according to the median value of risk scores. The Kaplan–Meier method was used to estimate the overall survival for the two groups, and differences in survival were analysed using the Log-rank test.

Declarations

Acknowledgements

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Author Contributions

X.L., J.X., and Y.L. conceived of the project. T.S., Y.X., and J.S. designed and performed the research, C.Y. and H.Z. performed the function enrichment analyses. J.X., Y.L., and X.L. supervised research and provided critical advice on the study. T.S. and Y.L. wrote the manuscript, with input from other co-authors. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Data availability

The paired lncRNA-mRNA expression profiles as well as DNA methylation and copy number data were obtained from the TCGA Data Portal (https://portal.gdc.cancer.gov/). The RNA-seq data of immune cells was downloaded from GEO under accession number GSE26530, GSE30811, GSE33772, GSE34260, GSE36952, GSE40131, GSE40548, GSE40718, GSE45734, GSE45982, GSE53419, GSE55320, GSE55536, GSE56179, GSE57494, GSE58596, GSE59846, GSE60482, GSE64182, GSE64655, GSE64713, GSE66117, GSE66385, GSE66763, GSE66895, GSE68482, GSE68795 and GSE72502. The Biological Process (BP) terms for Gene Ontology (GO) were downloaded from the MSigDB (v5.1) database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). Cancer-related lncRNAs were obtained from lnc2cancer 2.0 (http://www.bio-bigdata.net/lnc2cancer/).

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Figures

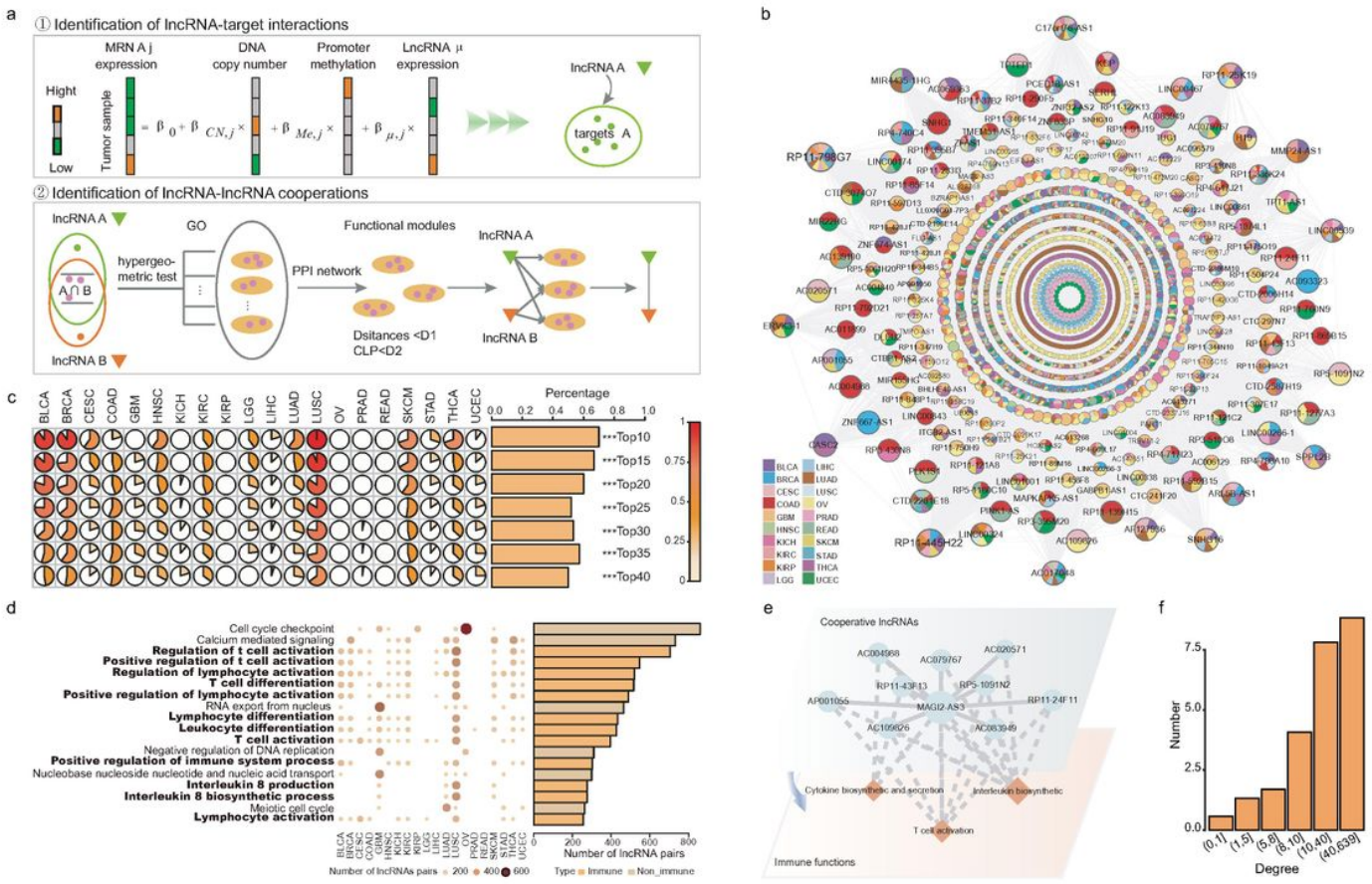

Figure 1

Cooperative lncRNAs contribute to immune-related functions. (a) A work flow used for identifying lncRNA-lncRNA co-regulations. **(b)** The lncRNA-lncRNA cooperative regulation network in pan-cancer. A node indicates a lncRNA, a colour pie chart shows cancers in which the lncRNA occurred and size of the node shows the number of connected nodes. An edge indicates a cooperative regulation between lncRNAs. **(c)** The percentage of immune functions in the first few of all the functions in each cancer type and pan-cancer. **(d)** The bubble plot shows the number of lncRNA cooperative pairs regulating each function across cancer types. The right bar plot shows the total number of lncRNA pairs regulating each function in pan-cancer network. The functions in the black font and the dark yellow colour are immune-related functions. **(e)** An example of a sub-network which consists of cooperative lncRNAs and regulated immune functions in LUSC. **(f)** The number of immune functions co-regulated by lncRNAs connected by different number of nodes.

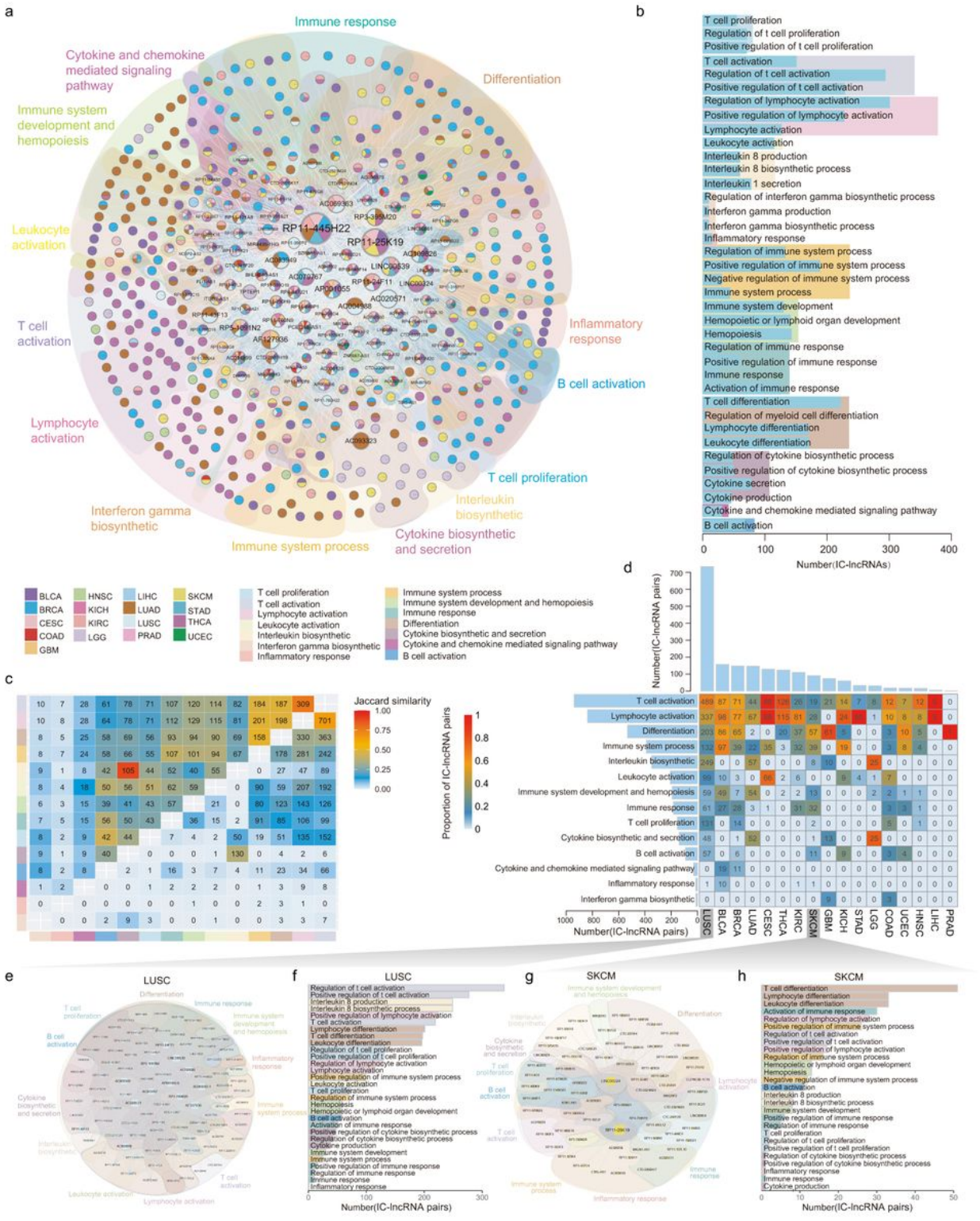

Figure 2

Landscape of lncRNA immune-related co-regulated networks. (a) The LICN in pan-cancer. The shadows in different colours represent different immune functions. **(b)** The number of IC-lncRNAs co-regulating immune-related functions. These immune functions are summarised into 14 categories. **(c)** The upper triangular matrix shows the proportion of IC-lncRNAs shared by any two immune function categories and the integer shows the number of shared IC-lncRNAs. The lower triangular matrix shows the proportion of

shared IC-lncRNA co-regulations. **(d)** The middle matrix shows the number of IC-lncRNA pairs co-regulating immune functions across cancer types and the color legend indicates the proportion of IC-lncRNA pairs co-regulating each immune function in each network. The top bar plot shows the number of IC-lncRNA pairs co-regulating all immune functions in each cancer. The left bar plot shows the number of IC-lncRNA pairs co-regulating each immune function in all 17 LICNs. **(e) and (g)** The landscape of IC-lncRNA cooperative regulations in LUSC and SKCM respectively. **(f) and (h)** The number of immune functions co-regulated by IC-lncRNA cooperative regulations in LUSC and SKCM respectively.

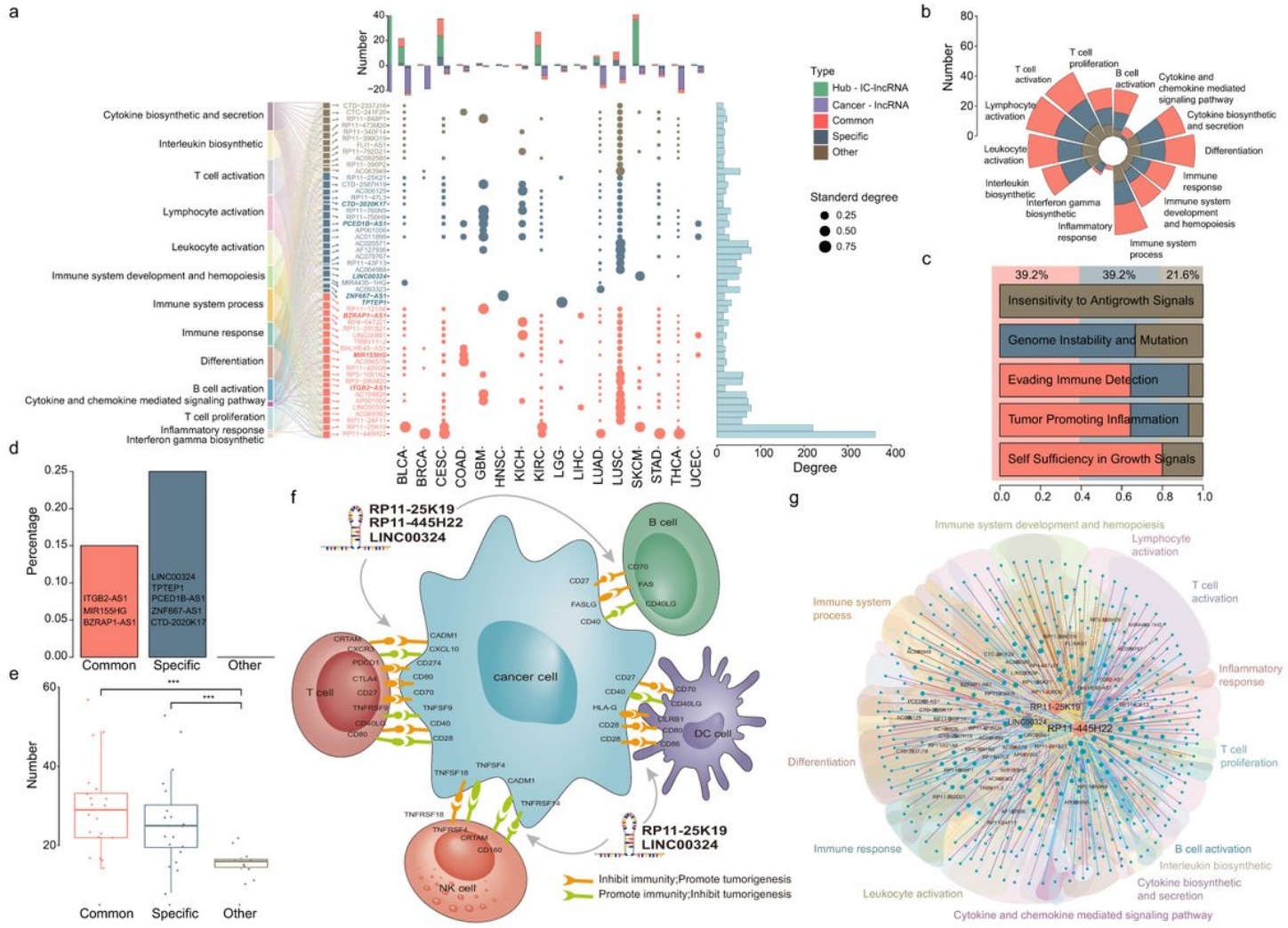

Figure 3

The conserved and rewired LICN hubs in each cancer type. (a) The classification of hub IC-lncRNAs in pan-cancer LICN. The top bar plot indicates the number of hub IC-lncRNAs and cancer-related IC-lncRNAs in LICNs of cancers. The right bar plot shows the number of IC-lncRNAs connected with each hub IC-lncRNA in the LICN. The middle bubble diagram indicates the number of IC-lncRNAs connected with each hub IC-lncRNA divided by the total number of IC-lncRNAs in each LICN. **(b)** The number of different kinds of hub IC-lncRNAs co-regulating each immune function category. **(c)** The proportion of hub IC-lncRNAs

co-regulating each cancer hallmark in each hub category. The colored shadow shows the proportion of three kinds of all hub IC-lncRNAs. **(d)** The proportion of cancer-related hub IC-lncRNAs in each hub category. The cancer-related hub IC-lncRNAs are shown in the plot. **(e)** The number of drug target genes regulated by different kinds of hub IC-lncRNAs. Two-sided t test was used, *** p<0.001. **(f)** A diagram shows the interactions between T cells and tumor cells modulated by three IC-lncRNAs. The target genes regulated by IC-lncRNAs showed in the plot are receptors and ligands expressed on the surface of immune cells or tumor cells. **(g)** An immune cooperative regulation sub network consisting of IC-lncRNA cooperative regulations where the three hub IC-lncRNAs showed in figure f involved.

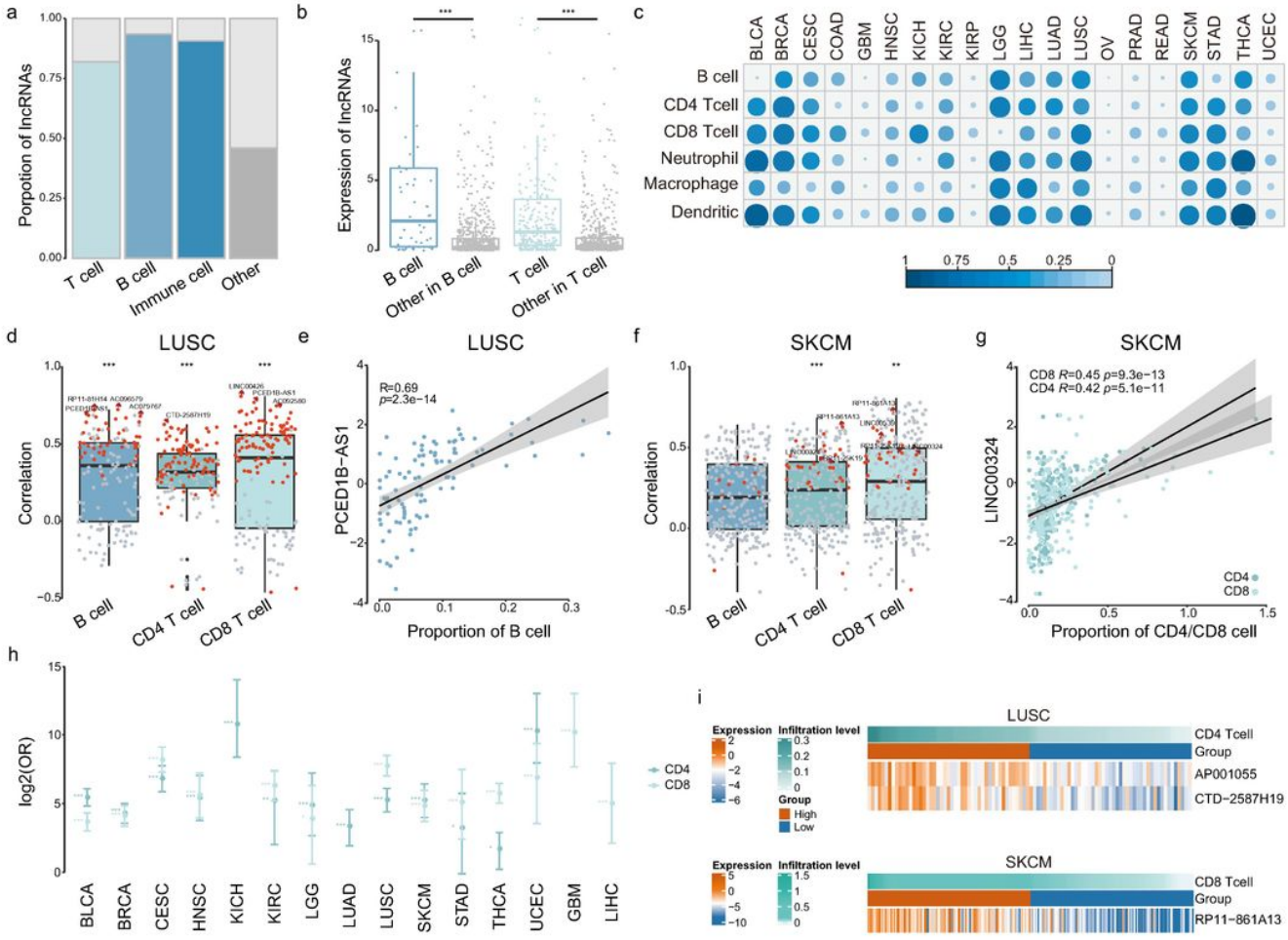

Figure 4

IC-lncRNAs were correlated with immune cell (B cells and T cells) infiltration. (a) The proportion of IC-lncRNAs expressed in immune cells from GEO RNA expression profiles. The genes which expression was zero in more than 80% of samples were abandoned. **(b)** The differences in expression between the IC-lncRNAs and other lncRNAs in immune cells using the GEO data were calculated by wilcoxon test. **(c)** The proportion of cooperative lncRNAs of which expressions are significantly related to immune cell infiltration across cancer types. The Spearman's rank correlation coefficients were calculated between the

expression of cooperative lncRNAs and the immune cell infiltration and cut-off is |R| > 0.3 and p < 0.05. **(d) and (f)** The significant Spearman's rank correlation coefficients between the expression of cooperative lncRNAs and immune cell infiltration in LUSC and SKCM respectively. A red dot represents an IC-lncRNA co-regulating immune cell-related functions. **(e) and (g)** Examples of two IC-lncRNAs closely related to immune cell infiltration. **h** The plot shows the odds ratios in each cancer type. The significance of overlap between lncRNAs differentially expressed in the high or low immune cell infiltration group and IC-lncRNAs co-regulating T cell-related functions was calculated by two-sided Fisher`s exact tests. The error bars were 95% confidence levels of odds ratios. **I** The plot shows the significantly differentially expressed T cell-related IC-lncRNAs between high infiltration group and low infiltration group.

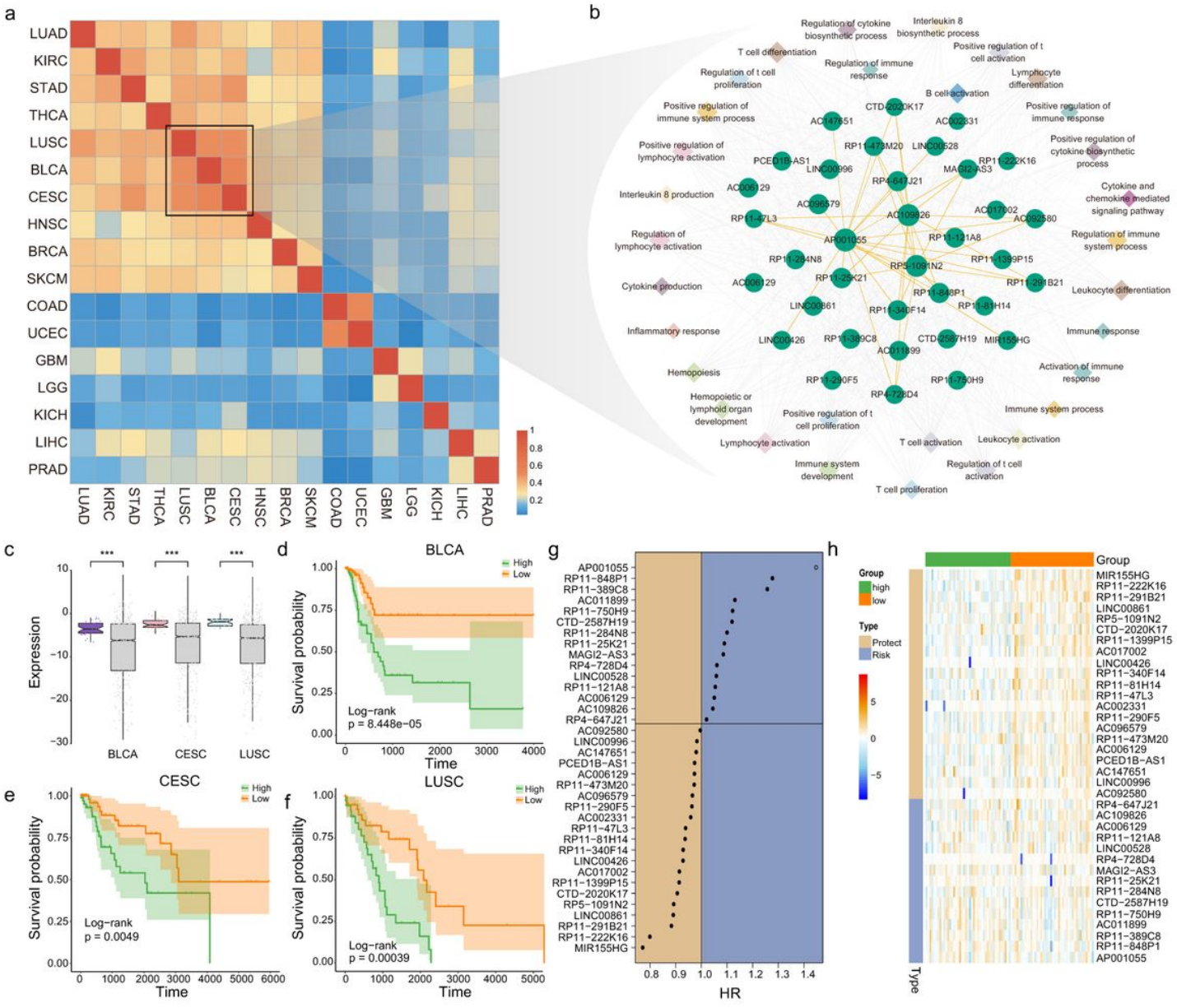

Figure 5

IC-lncRNA cooperation reveals similar regulation of cancer immune microenvironment. (a) Cancer clusters identified by hierarchical clustering based on the mean of Jaccard coefficients calculated by the number of shared IC-lncRNAs divided by the size of the union set plus expression correlations of pan-cancer IC-lncRNAs. **(b)** The sub-network consisting of IC-lncRNAs shared by BLCA, CESC and LUSC and co-regulated functions. A circle shows an IC-lncRNA and a diamond shows an immune function. The same colour diamond shows immune functions belonging to the same function category. An edge in orange represents a cooperative regulation between IC-lncRNAs; an edge in grey represents a regulation between IC-lncRNAs and immune functions. **(c)** The expression of IC-lncRNAs shared by BLCA, CESC and LUSC. The grey box shows the expression of other lncRNAs in each cancer. Two-sided t test was used. *** $p<0.001$. **(d-f)** The differences in survival between groups classified by the median of risk scores based on the IC-lncRNAs shared by these three cancer types in each cancer. **(g)** The hazard ratios (HR) of IC-lncRNAs shared by three cancer types according to univariate cox analysis in LUSC. A IC-lncRNA of which HR is larger than 1 is a risky IC-lncRNA, otherwise, a IC-lncRNA is a protective IC-lncRNA. **(h)** The expression of risky IC-lncRNAs and protective IC-lncRNAs in high-risk group and low-risk group.

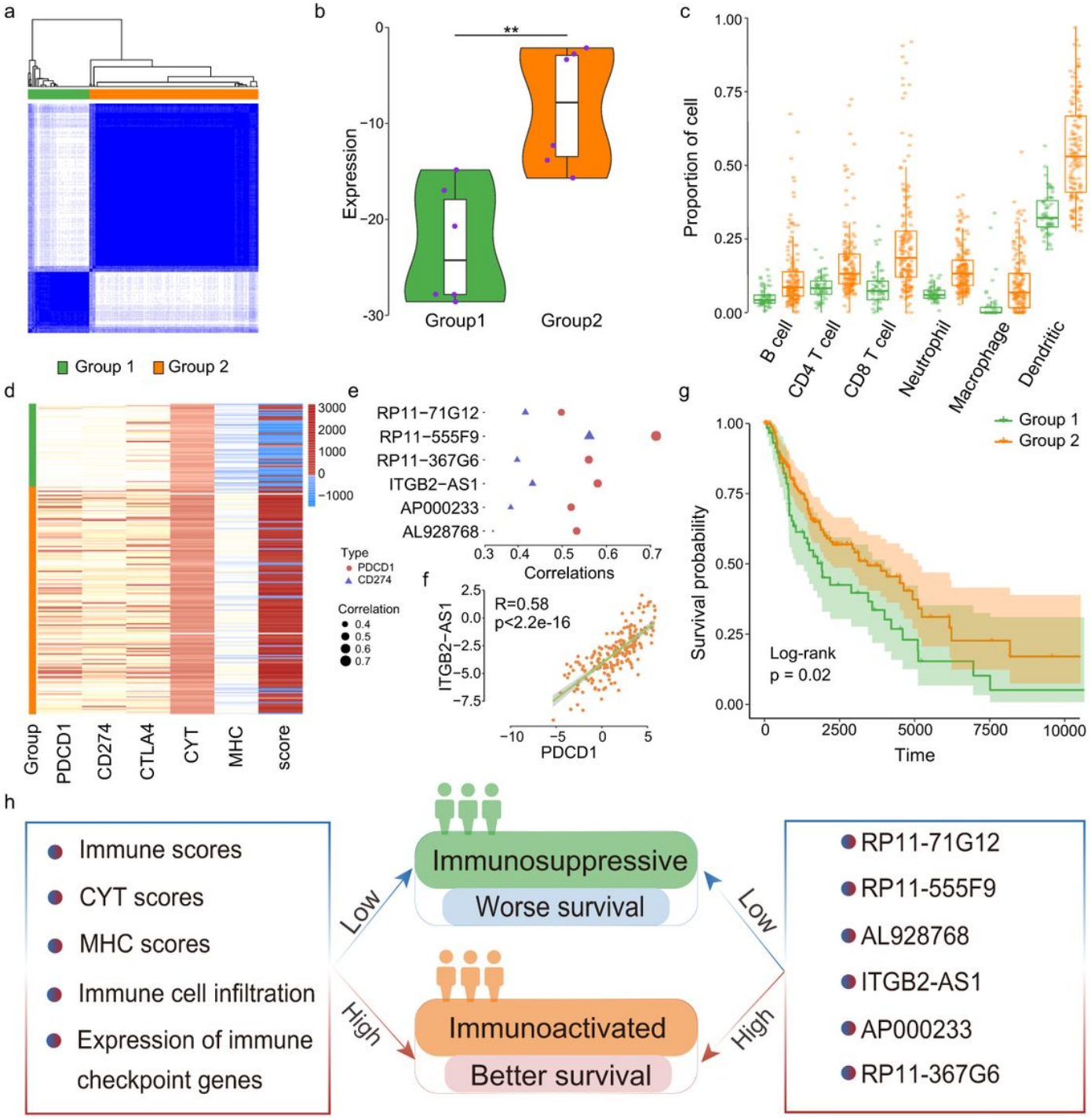

Figure 6

LICN contribute to subtype classification of SKCM. (a) Two immune subtypes identified by expression of seven immune hub IC-lncRNAs with high expression fluctuations using R package ConsensusClusterPlus in SKCM. **(b)** The expression of the six IC-lncRNAs in two immune subtypes. **(c)** The infiltration levels of six immune cells in two immune subtypes. Wilcoxon test was used, *** p<0.001. **(d)** The distribution of immune indicators (CYT, MHC and score (immune score)) and expression of immune checkpoint genes PDCD1, CD274, CTLA4 in two immune subtypes. **(e)** Pearson correlations between the expression of these IC-lncRNAs and PDCD1 or CD274. All p<0.05. **(f)** The expression of IC-lncRNA ITGB2-AS1 is closely

related to PDCD1. **(g)** Kaplan-Meier plot of survival for two immune subtypes in SKCM. The survival difference is calculated by log-rank test. **(h)** An illustration of two immune subtypes in SKCM. The immunoactivated subtype is characterized by higher immune cell infiltration levels, higher expression of immune indicators and immune checkpoint genes and better survival. In contrast, the immunosuppressed subtype has opposite phenotype.

Supplementary Files

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