RNA-seq and Single-Cell Transcriptome Analyses of TRAIL Receptors Gene Expression in Human Osteosarcoma Cells and Tissues

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ABSTRACT: Osteosarcoma (OS) is the most common primary cancer in the skeletal system, characterized by a high incidence of lung metastasis, local recurrence and death. Systemic treatment of this aggressive cancer has not improved significantly since the introduction of chemotherapy regimens, underscoring a critical need for new treatment strategies. TRAIL receptors have long been proposed to be therapeutic targets for cancer treatment, but their role in osteosarcoma remains unclear. In this study, we investigated the expression profile of four TRAIL receptors in human OS cells using total RNA-seq and single-cell RNA-seq (scRNA-seq). The results revealed that *TNFRSF10B* and *TNFRSF10D* but not *TNFRSF10A* and *TNFRSF10C* are differentially expressed in human OS cells as compared to normal cells. At the single cell level by scRNA-seq analyses, *TNFRSF10B, TNFRSF10D, TNFRSF10A* and *TNFRSF10C* are most abundantly expressed in endothelial cells of OS tissues among nine distinct cell clusters. Notably, in osteoblastic OS cells, *TNFRSF10B* is most abundantly expressed, followed by *TNFRSF10D, TNFRSF10A* and *TNFRSF10C*. Similarly, in an OS cell line U2-OS using RNA-seq, *TNFRSF10B* is most abundantly expressed, followed by *TNFRSF10D, TNFRSF10A* and *TNFRSF10C*. According to the TARGET online database, poor patient outcomes were associated with low expression of *TNFRSF10C*. These results could provide a new perspective to design novel therapeutic targets of TRAIL receptors for the diagnosis, prognosis and treatment of OS and other cancers.

KEYWORDS: TRAIL receptor, gene expression, scRNA-seq, osteosarcoma, bioinformatics

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Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents, and often has a second peak in incidence in patients who are over 50 years of age.[1,2] OS is originated from cells of osteoblastic lineages with the presence of mesenchymal stem cells (MSCs) and immature bone matrix or osteoid.[3] OS most commonly arises in the metaphyses of long bones such as the distal femur, proximal tibia or proximal humerus.[4] Patients usually suffer from pain and swelling, and the diagnosis is made by histopathology and imaging examinations including radionuclide scans, X-ray and magnetic resonance imaging (MRI) and positron emission tomography (PET) scan.[5-7] Initially, a high serum level of alkaline phosphatase (ALP) was used as an OS prognostic test, but the results were inconsistent particularly when comparing adults and children.[8] To date there are still no reliable serum biomarkers for OS. The treatment of OS is a combination of chemotherapy such as doxorubicin, methotrexate and cisplatin, as well as surgical procedures, which cure approximately 60-70% of patients.[9,10] The pathogenesis of OS remains unclear and controversial, consequently resulting in a barrier to the development of novel diagnostic biomarkers and prognostic markers.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL, also known as TNFSF10 or APO2 ligand, is a cytokine first reported to have apoptosis-inducing properties.[11-13] TRAIL binds to a family of TRAIL receptors, including two receptors that transduce the apoptotic signals DR4 (or TRAILR1) and/or DR5 (or TRAILR2) and two TRAIL decoy receptors that function to antagonize TRAIL-induced apoptosis.[14,15] Interestingly, TRAIL was found to induce apoptosis in tumour cells but not in normal cells.[16]

Previous studies have found that the TRAIL transcripts are constitutively expressed in a variety of human tissues,[12,13] and in NK cells.[15] TRAIL receptors were found to express at various levels between normal human bone cells (NHBC) and OS cells, which could contribute to the resistance and sensitivity of TRAIL-mediated apoptosis.[17-19] TRAIL receptors might

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determine to the resistance or sensitivity of TRAIL-mediated apoptosis in OS. However, the expression profile of TRAIL receptors in OS tumour-microenvironment needs to be investigated.

In this study, in order to understand the complex physiological and/or pathological roles of TRAIL receptors, we analysed the expression profiles of TRAIL receptors in the OS tumour-microenvironment using RNA-seq[20,21] and scRNA-seq,[22] as well as in the U2-OS cell line by RNA-seq. By this study we aim to undercover the gene expression profiles of TRAIL receptors in OS tumour-microenvironment. In addition, we analysed the survival outcome of OS patients in association with the gene expression levels of TRAIL receptors.

Materials and Methods

Multiple sequence alignment and phylogenetic tree analyses of TRAIL receptors

Multiple sequence alignment and phylogenetic tree of human TRAIL receptors proteins were conducted using bioinformatic tools based on uniport, https://www.uniprot.org/align.

GEO database analyses

To compare the gene expression profile, the plots were generated based on the GEO database with the dataset GSE 42352 available athttps://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42352.[20,21] GSE42352 contains genome-wide gene expression profiling of 118 different sources, including mesenchymal stem cells (MSCs), osteoblasts, osteosarcoma cell lines and osteosarcoma biopsy. The 15 sets of normalized gene expression data of MSCs and osteoblasts were defined as the normal group, and 113 sets of normalized gene expression data of the osteosarcoma biopsies and osteosarcoma cell lines were defined as the tumour group. Statistical difference was determined by Student's *t*-test, and $p < 0.05$ value is considered statistically significant.

Cell clustering analyses based on scRNAseq dataset

Cell clustering analyses are conducted based upon a previously published dataset available on GSE162454, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162454, which was generated through the single-cell RNA-seq (scRNA-seq) dataset of six osteosarcoma patients. Data quality control was accomplished by using the 'Seurat' package with a version 4.0.5 through R studio with a version 4.1.0. Batch effects among cells were managed by using the 'harmony' package with a version 0.1.0. Poor quality cells were excluded from the dataset as previously described. The uniform manifold approximation and projection (UMAP) analysis was performed to visualize diverse cell clusters (parameter dim = 1:30, resolution = 0.10) by using dimensional reduction plot (DimPlot, version 2.3.2). High-quality colour of the UMAP was achieved by using the 'ggsci' package with a version 2.9.

Data visualization for comparative analyses

The quantity of scRNA-seq was measured by Transcripts per Million (TPM) which represents the relative expression level of transcripts by the 'Seurat' package based on the total read counts by the length of each gene in kilobases. Heatmaps were produced to show the transcript expression of genes of interest in each cell cluster using log10 TPM values. For the inter-cluster comparison analyses, violet plots were used to highlight the expression profile of genes of interest among cell clusters. Statistical analyses were conducted via R studio and a p-value < 0.05 was referred to as statistically significant. Multiple comparisons with Wilcoxon Rank Sum Test among the cell clusters were also generated and provided as supplementary results.

RNA sequencing of U2-OS cell line

Human osteosarcoma cell line U2-OS was obtained from the American Type Culture Collection (ATCC) and cultured in McCoy's 5A medium with 10% fetal bovine serum and 1% penicillin-streptomycin in a 37°C ± 0.5°C, 5% CO_2 humidified incubator. The total RNA was isolated using TRIzol reagent (Invitrogen Corp, Carlsbad, CA, USA). The RNA samples were prepared according to the protocol from Takara SMARTer V2 (Takara Bio Inc., Mountain View, CA, USA). The libraries were sequenced on Illumina NovaSeq 6000 and an S4-300 cycle lane (150PE) with v1.5 sequencing protocol. Raw counts were summarized by the featureCounts v1.5.3 utility of the Subread package (version 2.12.0). Raw data of RNA-seq was then converted to TPM and normalized to Trimmed mean of M value (TMM) using edgeR package (version 3.40.0) through R studio (version 4.1.0). Student's *t*-test and One-way ANOVA were used to conduct statistical analysis.

TARGET-based survival data analyses

The Therapeutically Applicable Research to Generate Effective Treatments (TARGET)—OS RNA-Seq data analyses were performed as previously described.[23,24] Briefly, TARGET-OS RNA-Sequencing data were downloaded by R studio through Bioconductor packages of BiocManager (Bioconductor version of 3.12, https://www.bioconductor.org/). The RNA-Seq data were merged with patients' clinical information and with expressed genes. Kaplan-Meier survival analysis was performed using R's survival packages; survival, survminer and survdiff. K–M < 0.05 was used as a cut-off criterion for the survival-related gene at overall survival.

Results

Nomenclatures of TRAIL receptors

A summary of nomenclatures of TRAIL receptors is listed in Table 1. TNFRSF10A/TRAILR1 and TNFRSF10B/TRAILR2 are true receptors with apoptosis inducing ability, whereas

Table 1. TRAIL receptor nomenclatures.

PROTEIN NAMES	GENE NAMES	SYNONYMS	SYNONYMS	UNIPROT NO
Tumour necrosis factor receptor superfamily member 10A	TNFRSF10A	TRAILR1	DR4	O00220
Tumour necrosis factor receptor superfamily member 10B	TNFRSF10B	TRAILR2	DR5	O14763
Tumour necrosis factor receptor superfamily member 10C	TNFRSF10C	TRAILR3	DCR1	O14798
Tumour necrosis factor receptor superfamily member 10D	TNFRSF10D	TRAILR4	DCR2	Q9UBN6

TNFRSF10C/TRAILR3 and TNFRSF10D/TRAILR4 are decoy receptors with a lack of apoptosis inducing ability, as they contain a truncated consensus death domain motif.[25,26] Multiple sequence alignment revealed four receptor sequences share sequence homology at the N terminal region of proteins, and TNFRSF10A and TNFRSF10B have long intracellular domain at the C terminal whereas TNFRSF10C and TNFRSF10D show lack of intracellular domain at the C terminus (Figure 1a). Family tree analyses showed that TNFRSF10A is most closely related to TNFRSF10B, followed by TNFRSF10C and TNFRSF10D (Figure 1b).

Differential expression of TRAIL receptors based on the human OS dataset GSE42352

To compare the expression profile of TRAIL receptors, the gene expression plots of TRAIL receptors were generated using the GEO database with the dataset GSE42352. The results showed that the *TNFRSF10A* and *TNFRSF10C* transcripts were not differentially expressed between human OS cells and the control (Figure 2a and c), whereas *TNFRSF10B* and *TNFRSF10D* transcripts were found downregulated in human OS cells when compared with the control (Figure 2b and d). Interestingly, the *TRAIL* gene (*TNFSF10*) was found to be upregulated in human OS cells when compared with the control (Supplemental Figure 1).

Clusters of cell types in OS by single-cell RNA-seq and expression of TRAIL receptors

To examine the gene expression of TRAIL receptors in OS patient samples the scRNA-seq data were employed using six OS patient tumour samples with a total of 29,278 cells. The diversity of cell types in OS tissues was identified with nine distinct clusters by the UMAP, and the clusters included myeloid cell 1, osteoblastic OS cell, NK/T cell, myeloid cell 2, osteoclast, carcinoma-associated fibroblast (CAF), plasmocyte, endothelial cell and B cell (Figure 3a and b). Violin plots showed that TRAIL receptors are differentially expressed in OS cells, in which *TNFRSF10B* is most abundantly expressed, followed by *TNFRSF10D*, *TNFRSF10A* and *TNFRSF10C* (Figure 4a-e, Table 2, Supplement Tables 1–4).

By heatmap analysis, TRAIL receptors most abundantly expressed in endothelial cells among other cell types including myeloid cell 1, NK/T cell, myeloid cell 2, osteoclast, carcinoma-associated fibroblast, plasmocyte and B cells (Figure 5). To confirm if TRAIL receptors were expressed in endothelial cells, endothelial cell marker genes *EGFL7* and *EPCAM1* were used in the heatmap analysis. The results showed *EGFL7* and *EPCAM1* genes were expressed exclusively in endothelial cells, in line with the gene expression of TRAIL receptors, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF10C* and *TNFRSF10D* (Figure 5), indicating that TRAIL receptors are expressed in endothelial cells in OS patient samples. Similarly, the *TRAIL (TNFSF10)* gene was also found to be highly expressed in endothelial cells among other cell types (Figure 5, Table 2, Supplemental Figure 2). In comparison, *HIF1*α was found to be exclusively expressed in myeloid cell 1 in the heatmap analysis (Figure 5), indicating that myeloid cell 1 in OS is prone to a hypoxia condition as compared with other cells.

Expression of TRAIL receptors in an OS cell line U2-OS by RNA-seq

To examine the gene expression of TRAIL receptors in U2-OS, RNA-seq was performed. The results showed that TRAIL receptors were expressed at various levels, in which *TNFRSF10B* gene was most abundantly expressed followed by *TNFRSF10D*, *TNFRSF10A* and *TNFRSF10C* genes by TPM analysis (Figure 6). Endothelial cell marker gene *EPCAM1* was included as a reference and showed *EPCAM1* gene was barely expressed in U2-OS, whereas *EGFL7* showed an intermediate level of expression in U2-OS. In comparison, hypoxia marker gene *HIF1*α was moderately expressed in U2-OS by TPM analysis.

TARGET-based survival analyses of TRAIL receptors

To examine if the levels of TRAIL receptors gene expression in osteosarcoma samples are associated with patients' survival, TARGET-OS RNA-seq data was downloaded through R studio and merged with gene expressions. Kaplan-Meier survival analysis revealed that the expression levels of *TNFRSF10A*, *TNFRSF10B* and *TNFRSF10D* were not associated with a survival rate, whereas the low expression level of *TNFRSF10C* was associated with a worse survival outcome in OS patients (Figure 7).

A

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SP|O14763|TR10B_HUMAN ---------------------------------------------MEQRG----------- 5
SP|O00220|TR10A_HUMAN MAPPPARVHLGAFLAVTPNPGSAASGTEAAAATPSKVWGSSAGRIEPRGGGRGALPTSMG 60
SP|Q9UBN6|TR10D_HUMAN ------------------------------------------------------MGLWG 5
SP|O14798|TR10C_HUMAN ---------------------------------------------------------- 

SP|O14763|TR10B_HUMAN QNAPAASGARKRHGPGPREARGARPGPRVPKTLVLVV--AAVLLLVSAESALITQQDLAP 63
SP|O00220|TR10A_HUMAN QHGPS-ARARAGRAPGPRPAREASPRLRVHKTFKFVV--VGVLLQVVPSSAATIK---LH 114
SP|Q9UBN6|TR10D_HUMAN QSVPTASSARAGRYPGARTASGTRPWLLDPKILKFVVFIVAVLLPVRVDSATIPRQDEVP 65
SP|O14798|TR10C_HUMAN -------------------------MARIPKTLKFVVVIVAVLLPVLAYSATTARQEEVP 35
                                               * : :**  ..*** *   **   :

SP|O14763|TR10B_HUMAN QQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDYSTHWNDLLFCLRCTRCDSGE 123
SP|O00220|TR10A_HUMAN DQSIGTQQWEHSPLGELCPPGSHRSEHPGACNRCTEGVGYTNASNNLFACLPCTACKSDE 174
SP|Q9UBN6|TR10D_HUMAN QQTVAPQQQRRSLKEEECPAGSHRSEYTGACNPCTEGVDYTIASNNLPSCLLCTVCKSGQ 125
SP|O14798|TR10C_HUMAN QQTVAPQQQRHSFKGEECPAGSHRSEHTGACNPCTEGVDYTNASNNEPSCFPCTVCKSDQ 95
                     :*  . ** . *     ** * * **    *  *. * .*:   *:   *: ** *.*.:

SP|O14763|TR10B_HUMAN VELSPCTTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMVKVGDCTPWSDIECVHKES 183
SP|O00220|TR10A_HUMAN EERSPCTTTRNTACQCKPGTFRNDNSAEMCRKCSRGCPRGMVKVKDCTPWSDIECVHKES 234
SP|Q9UBN6|TR10D_HUMAN TNKSSCTTTRDTVCQCEKGSFQDKNSPEMCRTCRTGCPRGMVKVSNCTPRSDIKCKNES- 184
SP|O14798|TR10C_HUMAN KHKSSCTMTRDTVCQCKEGTFRNENSPEMCRKCS-RCPSGEVQVSNCTSWDDIQCVEEF- 153
                      . * ** **:*.***: *:*::.:* ****.*   ** * *:* :**  .**:* .:

SP|O14763|TR10B_HUMAN GTKHSGEVPAVEETVTSSPGTPA----------------------------------- 206
SP|O00220|TR10A_HUMAN GNGHNIWVILVV------------------------------------------- 246
SP|Q9UBN6|TR10D_HUMAN AASSTGKTPAAEETVTTILGMLA---------------------------------- 207
SP|O14798|TR10C_HUMAN GANATVETPAAEETMNTSPGTPAPAAEETMNTSPGTPAPAAEETMTTSPGTPAPAAEETM 213
                     .   .  .  .

SP|O14763|TR10B_HUMAN ------------------------SPCSLSGIIIGVTVAAVVLIVAVFVCKSLLWKKVLPY 243
SP|O00220|TR10A_HUMAN ----------------------------------TLVVP-----------LLLVAVLIV 260
SP|Q9UBN6|TR10D_HUMAN ----------------------SPYHYLIIIVVLVII--LAVVVVGFSCR----KKFISY 239
SP|O14798|TR10C_HUMAN TTSPGTPAPAAEETMITSPGTPASSHYLSCTIVGIIV--LIVLLIVFV------------ 259
                                                        :

SP|O14763|TR10B_HUMAN LKGICSGGGGDPERVDRS--------SQRPGAEDNVLNEIVSILQ--PTQVPEQEMEVQE 293
SP|O00220|TR10A_HUMAN CCCIGSGCGGDPKCMDRVCFWRLG-LLRGPGAEDNAHNEILSNADSLSTFVSEQQMESQE 319
SP|Q9UBN6|TR10D_HUMAN LKGICSGGGGGPERVHRVLFRRRSCPSRVPGAEDNARNETLSNRYLQPTQVSEQEIQGQE 299
SP|O14798|TR10C_HUMAN ---------------------------------------------------------- 

SP|O14763|TR10B_HUMAN PAEPTGVNMLSPGESEHLLEPAEAERSQRRRLLVPANEGDPTETLRQCFDDFADLVPFDS 353
SP|O00220|TR10A_HUMAN PADLTGVTVQSPGEAQCLLGPAEAEGSQRRRLLVPANGADPTETLMLFFDKFANIVPFDS 379
SP|Q9UBN6|TR10D_HUMAN LAELTGVTVESPEEPQRLLEQAEAEGCQRRRLLVPVNDADSA------------------ 341
SP|O14798|TR10C_HUMAN ---------------------------------------------------------- 

SP|O14763|TR10B_HUMAN WEPLMRKLGLMDNEIKVAKAEAAGHRDTLYTMLIKWVNKTGRDASVHTLLDALETLGERL 413
SP|O00220|TR10A_HUMAN WDQLMRQLDLTKNEIDVVRAGTAGPGDALYAMLMKWVNKTGRNASIHTLLDALERMEERH 439
SP|Q9UBN6|TR10D_HUMAN ---------------------------------------------DISTLLDASATLEEGH 357
SP|O14798|TR10C_HUMAN ---------------------------------------------------------- 

SP|O14763|TR10B_HUMAN AKQKIEDHLLSSGKFMYLEGNADSAMS-- 440
SP|O00220|TR10A_HUMAN AREKIQDLLVDSGKFIYLEDGTGSAVSLE 468
SP|Q9UBN6|TR10D_HUMAN AKETIQDQLVGSEKLFYEEDEAGSATSCL 386
SP|O14798|TR10C_HUMAN ----------------------------
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B

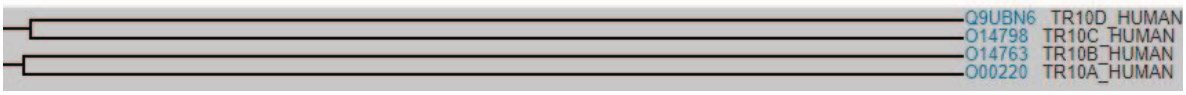

Figure 1. (a) Multiple sequence alignment of four TRAIL receptor protein sequences showing sequence homology at the N terminal region and (b) family tree analyses show that *TNFRSF10A* is most closely related to *TNFRSF10B*, followed by *TNFRSF10C* and *TNFRSF10D*.

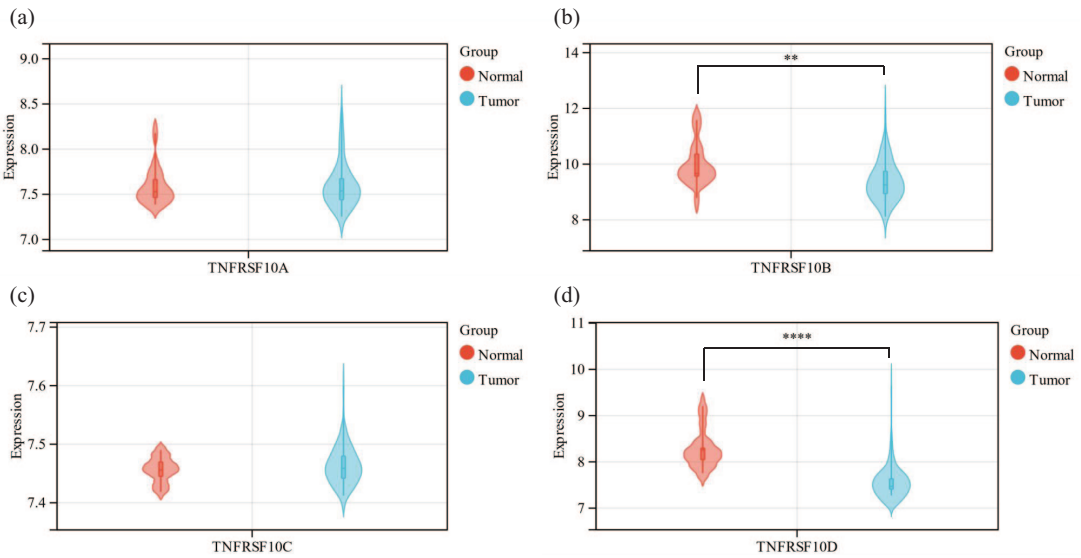

Figure 2. Violin plots of the TRAIL receptors genes expression levels: (a) *TNFRSF10A*, (b) *TNFRSF10B*, (c) *TNFRSF10C* and (d) *TNFRSF10D.* The violin plots filling in red representing normal groups, and the blue plots represent OS groups. The y-axis indicates the expression level of the genes. *P*-value was calculated using Student's *t*-test, $P < .05$ considered statistically significant (**$P < .01$, ****$P < .00001$).

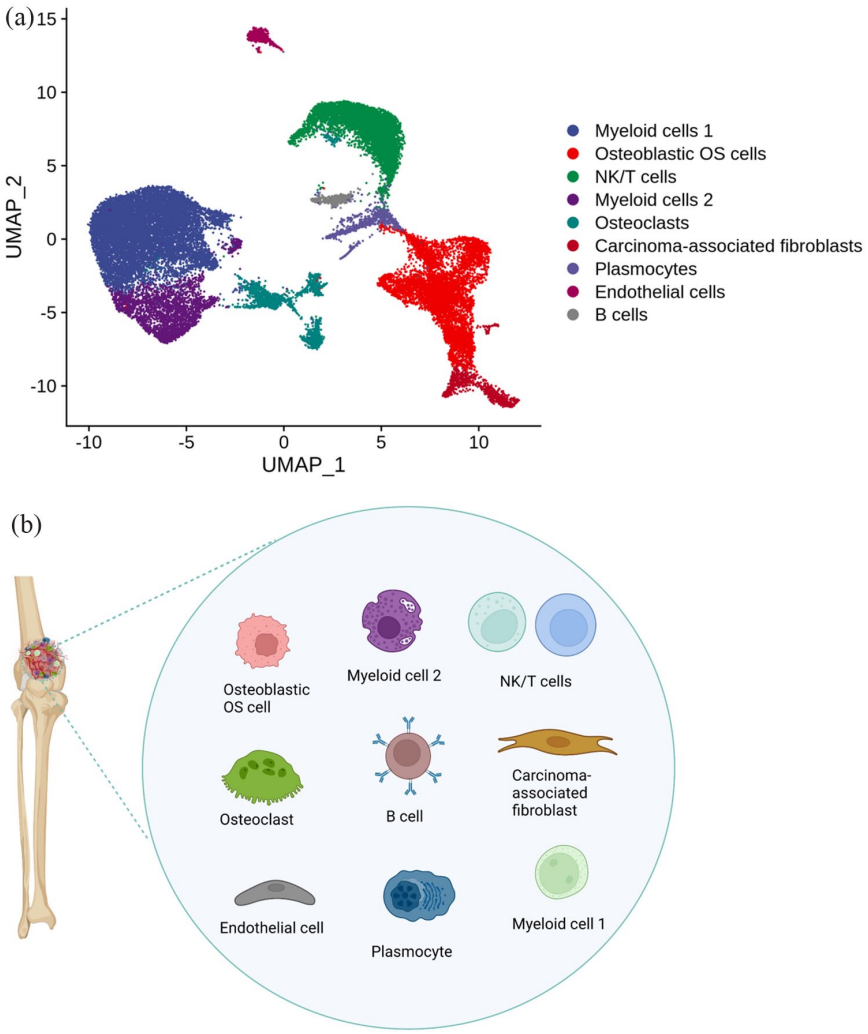

Figure 3. (a) Cellular heterogeneity of the OS with nine cell types demonstrated by Uniform manifold approximation and projection (UMAP) plot. Note that a 2-D UMAP plot with colour-coded cell clusters are shown and (b) diagram showing names of nine diverse cell types of OS including myeloid cell 1, osteoblastic OS cell, NK/T cell, myeloid cell 2, osteoclast, carcinoma-associated fibroblast (CAF), plasmocyte, endothelial cell and B cell and their putative location in the bone microenvironment.

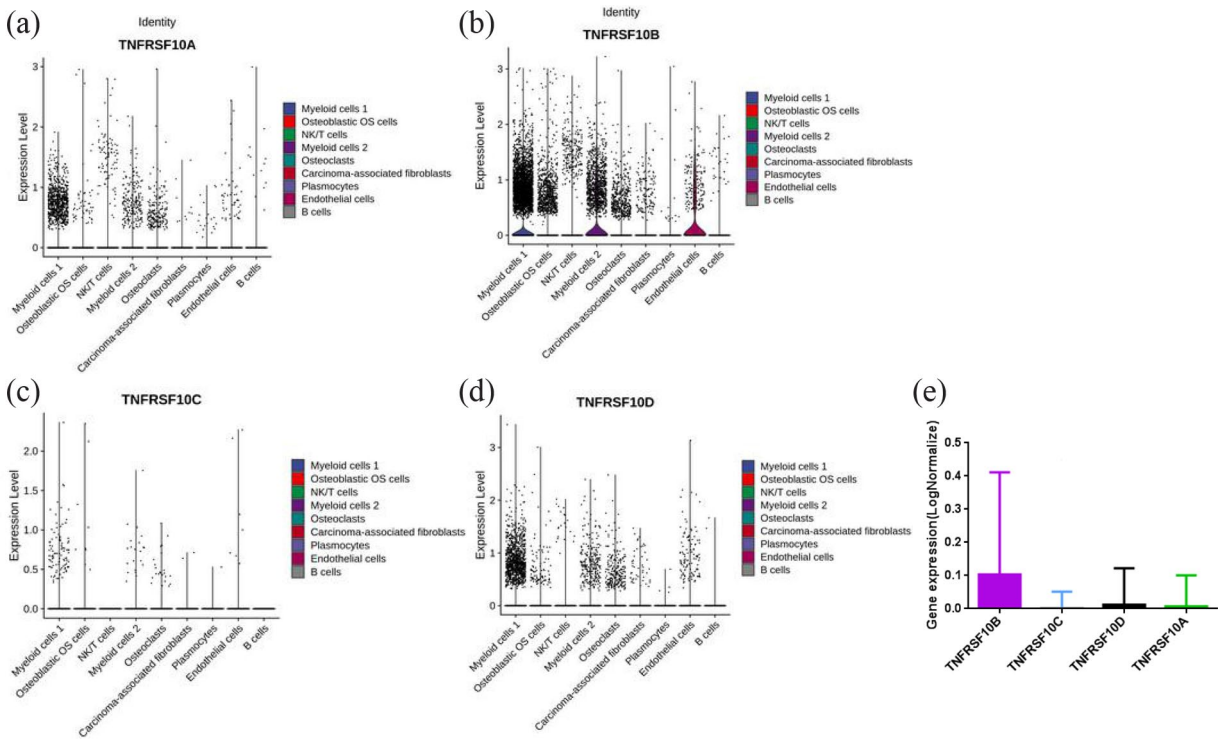

Figure 4. The violin plots show the average expression level of *TNFRSF10A* (a), *TNFRSF10B* (b), *TNFRSF10C* (c) and *TNFRSF10D* (d) in nine cell clusters in OS, including myeloid cell 1, osteoblastic OS cell, NK/T cell, myeloid cell 2, osteoclast, carcinoma-associated fibroblast (CAF), plasmocyte, endothelial cell and B cell. (e) Comparative analysis of the relative expression values of *TNFRSF10A, TNFRSF10B, TNFRSF10C* and *TNFRSF10D* is shown.

Discussion

In this study, RNA-seq revealed that *TNFRSF10B* and *TNFRSF10D* but not *TNFRSF10A* and *TNFRSF10C* were downregulated in human OS cells. Further, using scRNA-seq of OS,[22,23] the gene expression of TRAIL receptors was analysed using six human OS tissues. Surprisingly, TRAIL receptors were found mainly expressed in endothelial cells but weakly expressed in osteoblastic OS cells and other cell clusters. Since it was suggested that upregulating TRAIL receptors could be novel therapeutic approach for osteosarcoma, these data might guide us to understand the differential expression of TRAIL receptors in a cell type specific manner, paving the way for selective TRAIL receptor-mediated therapy in OS.

TRAIL receptors consist of two receptors that transduce the apoptotic signals DR4 (TRAILR1 or TNFRSF10A) and/or DR5 TRAILR2 or TNFRSF10B) and two TRAIL decoy receptors that function to antagonize TRAIL-induced apoptosis.[14,15] The induction of apoptosis by TRAIL likely requires oligomerization of the receptor,[26] which induce the adapter molecule FADD to recruit caspase-8 and death-inducing signalling complex (DISC) to mediate apoptosis.[27] Both receptors can interact with FADD, TRADD and RIP and activate the signalling of NF-kappa-B.[28] TRAIL also binds TNFRSF10C/TRAILR3[29] which lack a cytoplasmic death domain and hence is not capable of inducing apoptosis, and bind to TNFRSF10D/TRAILR4 which contains a truncated consensus cytoplasmic death domain motif.[30,31] Binding of TRAILR4 does not result in an apoptotic signal as TRAILR4 was not capable of inducing apoptosis but antagonize TRAIL-induced apoptosis,[32] whereas overexpression of TRAILR4 could protect cells bearing TRAILR1 and/or TRAILR2 from TRAIL-mediated apoptosis.[32] TRAIL also exhibited low affinity with Osteoprotegerin (OPG),[33] which might influence its effect on TRAIL receptors.[34] TNFRSF11B/OPG acts as a decoy receptor for TNFSF11/RANKL and inhibit RANKL -mediated osteoclastogenesis in the bone microenvironment.[35] Thus, TRAIL could block the anti-osteoclastogenic activity of OPG,[34] which might also contribute to the effect of TRAIL on OS cells. Taken together, the expression profiling and proportion of TRAIL receptors in osteosarcoma cells and other cells in the tumour microenvironment might help us to improve TRAIL receptor-mediated therapy through overcoming the resistance of OS cells by the design of druggable TRAIL.

To date, however, no clinical trial has been conducted for TRAIL in OS patients, and it remains to be seen if TRAIL could also affect OS cells. However, recombinant human TRAIL or agonistic monoclonal antibodies against DR4/5 have been used in phase 2 clinical studies but failed to show clinical efficacy.[36,37] Other studies have found that only some cancer types are responsive to TRAIL, while most tumours were resistant to TRAIL.[38,39] This property limits the potential of TRAIL-based cancer therapy. It would be interesting to determine if the expression profiling and proportion of TRAIL receptors are different in these cell types. In addition,

Table 2. The average expression level of *TRAIL* (*TNFSF10*) and TRAIL receptors among nine cell types of OS cells.

	MYELOID CELLS 1	OSTEOBLASTIC OS CELLS	NK/T CELLS	MYELOID CELLS 2	OSTEOCLASTS	CARCINOMA ASSOCIATED FIBROBLASTS	PLASMOCYTES	ENDOTHELIAL CELLS	B CELLS
TNFSF10	0.65737492	0.106174889	1.20198048	1.163070468	0.505474770	0.475266757	0.2463197182	1.86566419	0.21411995
TNFRSF10B	0.47348208	0.197918249	0.17163745	0.574878120	0.271906566	0.167890149	0.0430198284	0.54395216	0.19283730
TNFRSF10C	0.01234795	0.004276577	0.00000000	0.009091935	0.009229007	0.001787203	0.0007214498	0.04074832	0.00000000
TNFRSF10D	0.16081972	0.023688022	0.01510714	0.134076551	0.132772892	0.0021011117	0.0021011117	0.41387044	0.01603097
TNFRSF10A	0.09567656	0.017371129	0.06815942	0.096942798	0.105485460	0.0141883788	0.0141883788	0.15845970	0.11287938

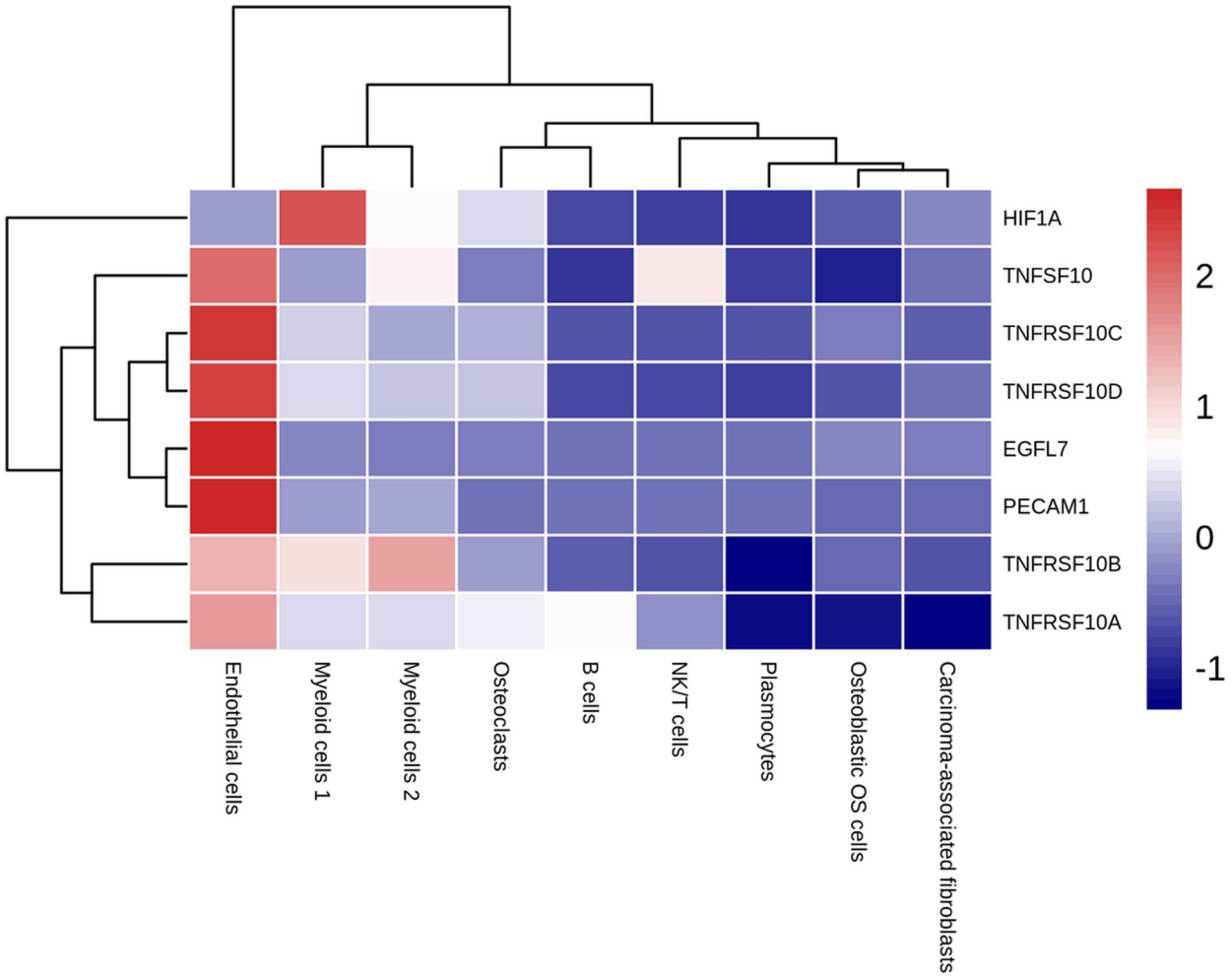

Figure 5. The heatmap analysis showing the differential expression of. Pink colour indicates higher expression, whereas light blue colour denotes lower expression. *TRAIL (TNFSF10), TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, EGFL7, EPCAM1* and *HIF1*α were included for comparison.

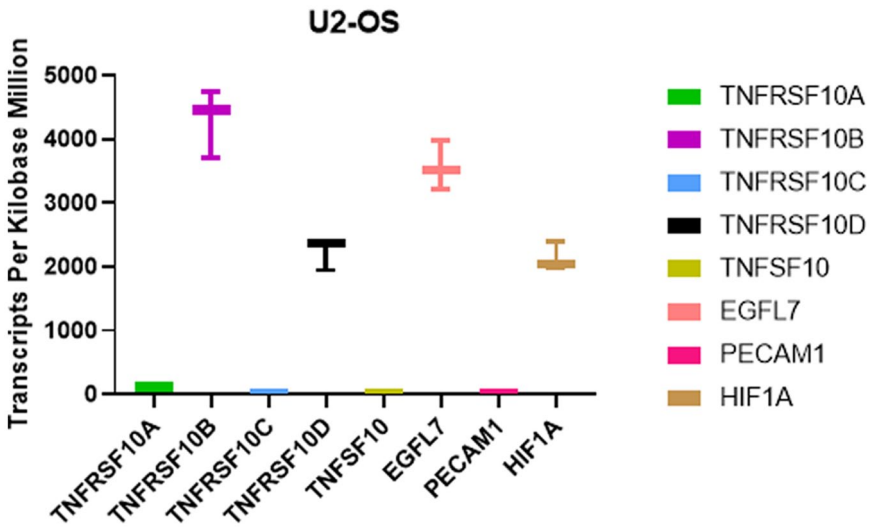

Figure 6. RNA-seq results showing the expressions of *TRAIL (TNFSF10)* and *TRAIL* receptors in OS cell line U2-OS by TPM. RNA-seq results of the expressions of *EGFL7, EPCAM1* and *HIF1a* were included for comparison.

TRAIL-resistance of cancer cells might be related to the expression or activity of c-FLIP and IAPs.[40] Inhibition of the IAPs activity and the c-FLIP expression was found to enhance TRAIL-induced apoptotic effects on cancer cells but does not affect normal cells.[40] Consistently, chemotherapeutic agents such as doxorubicin, cisplatin and etoposide could

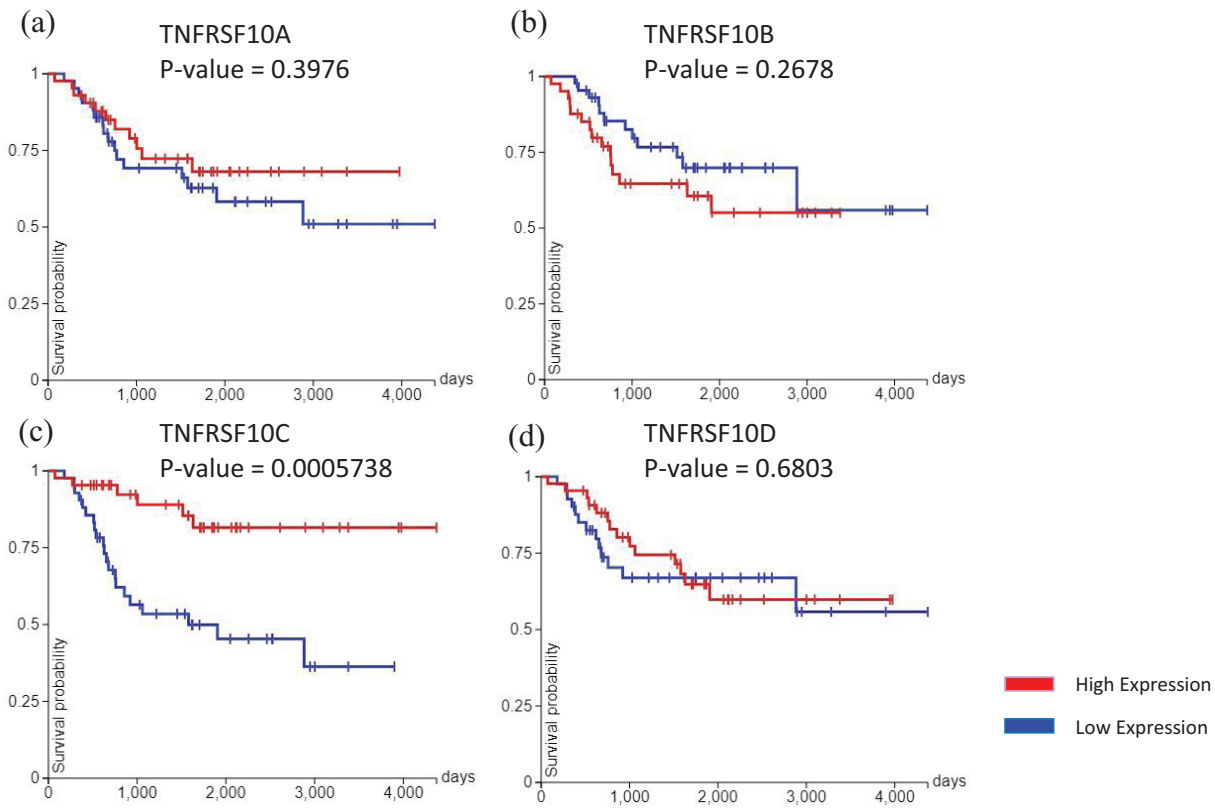

Figure 7. Kaplan-Meier survival analysis of OS patients' survival rate in association with the gene expression levels of *TNFRSF10A* (a), *TNFRSF10B* (b), *TNFRSF10C* (c) and *TNFRSF10D* (d). Red colour represents genes with high expression, whereas blue colour represents genes with low expression; *P*-value was computed by log-rank test, $P < .05$ considered statistically significant.

sensitize the response of OS cell line BTK-143 cells to Apo2L/TRAIL, with increased mRNA levels of DR4 and DR5.[19,41] Our RNA-seq analyses revealed that *TNFRSF10B* and *TNFRSF10D* but not *TNFRSF10A* and *TNFRSF10C* were downregulated in human OS cells, which might result in an unfavourable response of these cells to TRAIL. Notably, the *TRAIL (TNFSF10)* gene was found to be upregulated in human OS cells (Supplemental Figure 1). Further, we observed that the low expression level of *TNFRSF10C*, but not *TNFRSF10A, TNFRSF10B* and *TNFRSF10D* is associated with a low survival-rate in OS patients using Kaplan-Meier survival analysis in the TARGET-based survival data. In comparison, based on the Human Protein Atlas data analyses, high expression of *TNFRSF10A, TNFRSF10B* and *TNFRSF10D* is unfavourable in patients' survival in pancreatic cancer, renal cancer and cervical cancer; respectively (Supplement Figure 3 A-C), whereas data regarding the expression of *TNFRSF10C* in patients' survival in cancer is not available. Notably, high expression of *TRAIL* gene (*TNFSF10*) is unfavourable in patients' survival in pancreatic cancer (Supplement Figure 3D). These data suggest the complexity and versatility of the association between the expression levels of TRAIL receptors and patients' survival in various types of cancer. Further understanding how the expression of TRAIL receptors in a tissue type specific manner and their downstream signalling molecules will be essential for developing TRAIL-mediated therapy for OS and other cancers, as well as diagnosis and prognosis of cancers.

Recent studies have proposed that dysregulation of angiogenesis in the bone microenvironment could contribute to the pathogenesis of osteosarcoma and thus might serve as a therapeutic target.[42,43] Consistently, OS cells expressed abundant angiogenic genes such as *VEGF* and *EGFL7*.[44] Targeted anti-angiogenic therapies include bevacizumab, a monoclonal antibody to VEGF, and Endostar, a human recombinant endostatin as well as PDGF/PDGFR pathway inhibitors might represent new way of treating OS.[42,43] However, targeted anti-angiogenic regimens are still in its infancy and could be a long way from clinical applications for the disease. In this study, we found that *TNFRSF10B, TNFRSF10D, TNFRSF10A* and *TNFRSF10C* are most abundantly expressed in endothelial cells of OS tissues, with low levels of expression in osteoblastic OS cells among nine distinct cell clusters, which raise a possibility of endothelial cells and angiogenesis would also be a prime target for the TRIAL-mediated therapy for OS.

Authors' contributions

WF, HL and JX contributed to the preparation of paper. WF, HL, ER, DS, WZ, TN and QW contributed figure construction and data analyses. JZ, DW, YL and JX discussed and revised the paper. YL and JX conceptualized, supervised the studies and data collections and revised paper.

Data availability statement

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

Supplemental material

Supplemental material for this article is available online.

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