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# Impact of the cytotoxic T-lymphocyte associated antigen-4 rs231775 A/G polymorphism on cancer risk

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ABSTRACT

*Background:* Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) is an immunosuppressive checkpoint that is involved in the development and metastasis of cancers. Several studies revealed that CTLA-4 rs231775A/G polymorphism may be associated with the risk of cancer in some populations, but the conclusions of these studies are not consistent.

*Methods:* We conducted a pooled analysis with eligible studies to explore the association between the CTLA-4 rs231775 variant and cancer risk. Additionally, we used *in silico* tools to evaluated the expression of CTLA-4 on urinary system cancer. Moreover, we adopted the enzyme-linked immunosorbent assay (ELISA), and Gene Set Enrichment Analysis (GSEA) to investigate the effects of CTLA-4 on bladder cancer (BLCA).

*Results:* In total, 92 case-control studies involving 29,987 patients with cancer and 36,484 healthy individuals (controls) were included in the pooled analysis. In the stratified analysis based on cancer type, the rs231775 A/G polymorphism was associated with increased bladder cancer risk in the heterozygote contrast model (OR = 1.23, 95% CI = 1.01–1.51, $P = 0.040$). The race-stratified analysis revealed that East Asians with the GG genotype had a 12% lower risk of developing cancer than those with the GA + AA genotype (95% CI = 0.81–0.95, $P = 0.001$). The *in silico* analysis showed that CTLA-4 expression was augmented in patients with BLCA. The ELISA results revealed that CTLA-4 expression was reduced in patients with BLCA carrying the AA genotype. Several signaling pathways, including cytokine-cytokine receptor interactions and T-cell receptor signaling, were associated with CTLA-4 expression.

*Conclusion:* The CTLA-4 rs231775 A/G polymorphism is associated with cancer risk in East Asian population. This polymorphism is especially associated with BLCA.

## 1. Introduction

Cancer is a leading cause of death worldwide and is a major public health problem [1,2], with global statistical records indicating

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approximately 19.3 million new carcinoma cases and closed to 10 million cancer-related deaths in 2020 [1]. In Western Asia, the age-standardized rates of cancer incidence and mortality were 168.2 and 95.1 (per 100,000 individuals), respectively [3]. Although the overall trend of total carcinoma-related mortality was expected to diminish in China over the next decade, the cancer-related death toll (~3.0 million in 2020) is gradually increasing [4]. To date, no effective or specific biomarkers have been designed for numerous carcinomas [5,6]. Therefore, identifying specific molecular markers can help predict the occurrence and progression of malignant tumors and provide potential targets for cancer therapies [7].

Previous studies have shown that the immune system participates in the occurrence and development of cancer [8]. T lymphocytes are reportedly associated with the anti-tumor immune response in humans [9]. Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), formerly known as cluster of differentiation 152 (CD152), is a major immune checkpoint receptor protein that plays an essential role in regulating the anti-tumor immune response [10]. CTLA-4 is mainly expressed on the surface of T regulatory cells and activated T cells and belongs to the immunoglobulin superfamily [11]. CTLA-4 is an immunosuppressive checkpoint that can play a vital role in the initial stages of the immune response. Inhibition of immune checkpoint can slow down anti-tumor immune response and inhibit proliferation and metastasis of cancer cells. Additionally, CTLA-4 is an inhibitory receptor of immune cells and plays a pivotal role in maintaining the dynamic balance between B and T lymphocytes [12], and it inhibits the anti-tumor immune response by interfering with T-cell activation and thereby promoting cancer cell proliferation [13].

The CTLA-4 gene, located on human chromosome 2q33, comprises four exons [14]. Mutations in this gene can alter its expression and impact tumor development by mitigating anti-tumor immune activity and increasing the susceptibility of cells to malignancy [15,16]. Many cancer-related single nucleotide polymorphisms (SNPs) of the CTLA-4 gene have been reported, including rs231775 in exon 1; rs4553808, rs5742909, and rs733618 in the promoter region; and rs3087243 in the 3′ untranslated region [17,18]. The CTLA rs231775 polymorphism (+49A/G) has been extensively investigated [18] and is suggested to be associated with various cancers, including bladder cancer (BLCA), hepatocellular carcinoma, colorectal cancer, and cervical cancer [19–23]. However, because the results of these studies are still controversial [19], we conducted a comprehensive analysis of all eligible studies to better explain the association of the CTLA-4 rs231775 variant with cancer risk and different population types. Additionally, Gene Set Enrichment Analysis (GSEA), *in silico* tools, and the enzyme-linked immunosorbent assay (ELISA) were used to investigate the effects of CTLA-4 expression on patients with BLCA.

## 2. Materials and methods

### 2.1. Search strategy

An online literature search was conducted using the PubMed Central, Embase, Google Scholar, and Chinese Wanfang databases. The following keywords were included: ('rs231775' OR 'CTLA-4' OR 'cytotoxic T-lymphocyte associated antigen 4') AND ('malignant tumor' OR 'cancer') AND ('mutation' OR 'variant' OR 'polymorphism'). The last search was updated on December 30, 2022. Additionally, supplemental materials from the published articles were screened to ensure that all available genetic data were included.

### 2.2. Inclusion and exclusion criteria

The selection criteria were as follows: (a) case-control studies on the correlation between the CTLA-4 rs231775 A/G polymorphism and cancer risk; and (b) studies containing available genotype frequencies for measuring odds ratios (ORs) and manuscripts that could be obtained from the Internet. The exclusion criteria were as follows: (a) studies with data not concerning the CTLA-4 rs231775 A/G polymorphism and cancer risk; (b) case series lacking genetic data from the control group; and (c) comments or reviews with overlapping information.

### 2.3. Data extraction

Two independent researchers retrieved the basic information from each qualified report. The following factors were included: name of the first author, country of study, type of cancer, source of controls, race of the study population, number of cases and controls, genetic distribution of the rs231775 A/G polymorphism, *P*-value for Hardy-Weinberg equilibrium (HWE), and methods used for genotyping. If multiple studies were included in a single article, the data from those studies were recorded separately. As there was only one study each focusing on prostate cancer (PRAD), esophageal cancer, glioma, or thymoma, these diseases were classified into the "other cancers" group.

### 2.4. Statistical analyses

We used ORs and 95% confidence intervals (CI) to explore the strength of the reported correlations between CTLA-4 rs231775 A/G polymorphism and cancer risk. Five genetic models were conducted as follows: allelic comparison (G allele vs. A allele), heterozygote contrast (GA vs. AA), homozygous comparison (GG vs. AA), dominant (GG + GA vs. AA) and recessive model (GG vs. GA + AA). The heterogeneity of the included studies was measured using the *Q* statistic test. If the *P*-value of heterogeneity ($P_{\text{heterogeneity}}$) was less than 0.05, indicating that heterogeneity existed, then the random effects method (DerSimonian and Laird) was used; otherwise, the fixed effects method was applied. The *P*-value of HWE ($P_{\text{HWE}}$) in the control group was calculated using Fisher's exact test. A study with a $P_{\text{HWE}}$ value higher than 0.05 was classified into the "high-quality" group. Studies containing more than 1000 samples were classified

**Table 1**
Study characteristics of CTLA-4 rs231775 A/G polymorphism in the present analysis.

| First author | Year | Origin | Cancer | Ethnicity | Source | Case | Control | Case | | | Control | | | HWE | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs231775 A/G | | | | | | | | GG | GA | AA | GG | GA | AA | | |
| Kasamatsu | 2020 | Japan | Myeloma | East Asian | HB | 124 | 211 | 50 | 60 | 14 | 75 | 105 | 31 | 0.556 | PCR-RFLP |
| Mao | 2020 | China | Bladder | East Asian | HB | 354 | 434 | 21 | 127 | 206 | 14 | 132 | 288 | 0.812 | PCR-RFLP |
| Babteen | 2020 | Egypt | Breast | African | HB | 93 | 179 | 7 | 35 | 51 | 20 | 92 | 67 | 0.164 | TaqMan |
| Yang | 2019 | China | Liver | East Asian | PB | 575 | 920 | 290 | 221 | 64 | 444 | 389 | 87 | 0.893 | SNPscan Kit |
| Liu | 2019 | China | Gastric | East Asian | PB | 487 | 1470 | 228 | 215 | 44 | 698 | 631 | 141 | 0.926 | SNPscan Kit |
| Zou | 2018 | China | Colorectal | East Asian | PB | 979 | 1299 | 417 | 443 | 119 | 621 | 563 | 115 | 0.430 | SNPscan Kit |
| Wagh | 2018 | Indian | Cervical | South Asian | HB | 92 | 57 | 23 | 38 | 31 | 20 | 30 | 7 | 0.405 | PCR-RFLP |
| Abtahi | 2018 | Iran | Thyroid | South Asian | HB | 164 | 100 | 14 | 70 | 80 | 3 | 29 | 68 | 0.965 | PCR-RFLP |
| Qin | 2017 | China | Myeloma | East Asian | HB | 86 | 154 | 42 | 32 | 12 | 60 | 78 | 16 | 0.201 | TaqMan |
| Karabon | 2017 | Poland | Prostate | Caucasian | PB | 301 | 301 | 43 | 154 | 104 | 59 | 142 | 100 | 0.503 | PCR-RFLP |
| Chang | 2017 | China | Thyroid | East Asian | HB | 324 | 350 | 170 | 130 | 24 | 159 | 142 | 49 | 0.062 | PCR-RFLP |
| Chen | 2017 | China | Lung | East Asian | PB | 520 | 1028 | 254 | 219 | 47 | 504 | 431 | 93 | 0.950 | SNPscan Kit |
| Aldaiturriaga | 2017 | Spain | Bone | Caucasian | HB | 66 | 125 | 6 | 28 | 32 | 5 | 83 | 37 | 0.101 | PCR-RFLP |
| Isitmangil | 2016 | Turkey | Breast | Caucasian | HB | 79 | 76 | 6 | 24 | 49 | 6 | 36 | 34 | 0.402 | PCR-RFLP |
| Ma | 2015 | China | Lung | East Asian | HB | 528 | 600 | 74 | 282 | 172 | 72 | 306 | 222 | 0.031 | PCR-RFLP |
| Liu | 2015 | China | Liver | East Asian | HB | 80 | 78 | 29 | 36 | 15 | 38 | 33 | 7 | 0.966 | PCR-RFLP |
| Kucukhuseyin | 2015 | Turkey | Colorectal | Caucasian | PB | 80 | 115 | 6 | 36 | 38 | 9 | 52 | 54 | 0.467 | PCR-RFLP |
| Li | 2015 | China | Colorectal | East Asian | PB | 231 | 325 | 18 | 138 | 75 | 46 | 131 | 148 | 0.057 | PCR-RFLP |
| Liu | 2015 | China | Esophageal | East Asian | PB | 604 | 664 | 307 | 254 | 43 | 310 | 296 | 58 | 0.283 | PCR-LDR |
| Tang | 2015 | China | Gastric | East Asian | PB | 330 | 590 | 155 | 153 | 22 | 278 | 264 | 48 | 0.179 | PCR-LDR |
| Liu | 2015 | China | Lung | East Asian | HB | 231 | 250 | 77 | 101 | 53 | 51 | 91 | 108 | 0.059 | PCR-RFLP |
| Wang | 2015 | China | Colorectal | East Asian | HB | 311 | 289 | 121 | 147 | 43 | 141 | 47 | 101 | 0.001 | TaqMan |
| Ge | 2015 | China | Colorectal | East Asian | HB | 572 | 626 | 296 | 242 | 34 | 292 | 284 | 50 | 0.095 | PCR-RFLP |
| Tupikowski | 2015 | Poland | Renal | Caucasian | PB | 236 | 505 | 42 | 123 | 71 | 104 | 244 | 157 | 0.607 | TaqMan |
| Yu | 2015 | China | Breast | East Asian | PB | 376 | 366 | 27 | 175 | 174 | 35 | 157 | 174 | 0.962 | PCR-RFLP |
| Xiong | 2014 | China | Cervical | East Asian | HB | 365 | 421 | 35 | 189 | 141 | 72 | 181 | 168 | 0.056 | TaqMan |
| Minhas | 2014 | Indian | Breast | South Asian | NA | 250 | 250 | 26 | 113 | 111 | 24 | 121 | 105 | 0.197 | PCR-RFLP |
| Jaiswal | 2014 | Indian | Bladder | South Asian | HB | 212 | 200 | 16 | 79 | 117 | 6 | 57 | 137 | 0.981 | PCR-RFLP |
| Hui | 2014 | China | Leukemia | East Asian | HB | 86 | 112 | 36 | 41 | 9 | 55 | 42 | 15 | 0.137 | PCR-RFLP |
| Song | 2013 | China | Lung | East Asian | HB | 158 | 72 | 70 | 66 | 22 | 34 | 31 | 7 | 0.986 | PCR-RFLP |
| Cui | 2013 | China | Colorectal | East Asian | PB | 128 | 205 | 73 | 46 | 9 | 84 | 68 | 53 | <0.001 | PCR-RFLP |
| Wang | 2013 | China | Bladder | East Asian | HB | 300 | 300 | 15 | 90 | 195 | 31 | 91 | 178 | 0.005 | PCR-RFLP |
| Antczak | 2013 | Poland | Lung | Caucasian | HB | 71 | 104 | 2 | 13 | 56 | 22 | 33 | 49 | 0.001 | TaqMan |
| Gokhale | 2013 | Indian | Cervical | South Asian | HB | 104 | 162 | 26 | 46 | 32 | 48 | 87 | 27 | 0.239 | PCR-RFLP |
| Feng | 2013 | China | Bone | East Asian | PB | 308 | 362 | 110 | 150 | 48 | 155 | 179 | 28 | 0.055 | PCR-RFLP |
| Bharti | 2013 | Indian | HN | South Asian | HB | 130 | 180 | 0 | 63 | 67 | 25 | 80 | 75 | 0.622 | PCR-RFLP |
| Khorshied | 2013 | Egypt | Lymphoma | African | HB | 181 | 200 | 21 | 88 | 72 | 18 | 76 | 106 | 0.416 | PCR-RFLP |
| Queirolo | 2013 | Italy | Melanoma | Caucasian | HB | 14 | 45 | 0 | 8 | 6 | 3 | 16 | 26 | 0.802 | PCR-RFLP |
| Liu | 2013 | China | Lymphoma | East Asian | PB | 291 | 300 | 138 | 127 | 26 | 148 | 118 | 34 | 0.163 | PCR-LDR |
| Qi | 2012 | China | Gastric | East Asian | PB | 118 | 96 | 65 | 45 | 8 | 30 | 45 | 21 | 0.595 | PCR-RFLP |
| Fan | 2012 | China | Colorectal | East Asian | HB | 291 | 352 | 22 | 146 | 123 | 44 | 138 | 170 | 0.059 | PCR-RFLP |
| Erfani | 2012 | Iran | HN | South Asian | HB | 80 | 85 | 4 | 35 | 41 | 6 | 29 | 50 | 0.531 | PCR-RFLP |
| Yang | 2012 | China | Bone | East Asian | PB | 223 | 302 | 77 | 114 | 32 | 127 | 149 | 26 | 0.054 | PCR-RFLP |
| Lang | 2012 | China | Pancreatic | East Asian | PB | 602 | 651 | 208 | 312 | 82 | 263 | 326 | 62 | 0.056 | PCR-RFLP |
| Karabon | 2012 | Poland | Bone | Caucasian | PB | 199 | 368 | 48 | 103 | 48 | 75 | 169 | 124 | 0.213 | PCR-RFLP |
| Yang | 2012 | China | Pancreatic | East Asian | PB | 368 | 926 | 140 | 178 | 50 | 482 | 374 | 70 | 0.828 | PCR-RFLP |
| Li | 2012 | China | Breast | East Asian | PB | 576 | 553 | 246 | 281 | 49 | 256 | 243 | 54 | 0.739 | PCR-RFLP |

(*continued on next page*)

**Table 1** (*continued*)

| First author | Year | Origin | Cancer | Ethnicity | Source | Case | Control | Case | | | Control | | | HWE | Method |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs231775 A/G | | | | | | | | GG | GA | AA | GG | GA | AA | | |
| Li | 2011 | China | Cervical | East Asian | PB | 314 | 320 | 140 | 144 | 30 | 173 | 129 | 18 | 0.339 | PCR-RFLP |
| Karabon | 2011 | Poland | Lung | Caucasian | PB | 208 | 324 | 34 | 106 | 68 | 72 | 145 | 107 | 0.089 | PCR-RFLP |
| Liu | 2011 | China | Bone | East Asian | HB | 267 | 282 | 99 | 128 | 40 | 120 | 140 | 22 | 0.053 | PCR-RFLP |
| Wang | 2011 | China | Bone | East Asian | PB | 205 | 216 | 64 | 106 | 35 | 87 | 108 | 21 | 0.130 | PCR-RFLP |
| Wu | 2011 | China | Glioma | East Asian | HB | 653 | 665 | 297 | 259 | 97 | 300 | 295 | 70 | 0.841 | PCR-LDR |
| Cheng | 2011 | China | HN | East Asian | NA | 205 | 205 | 54 | 105 | 46 | 90 | 79 | 36 | 0.054 | PCR-RFLP |
| Jiang | 2011 | China | Cervical | East Asian | HB | 100 | 110 | 45 | 42 | 13 | 42 | 49 | 19 | 0.473 | PCR-RFLP |
| Gogas | 2010 | Greece | Melanoma | Caucasian | PB | 286 | 288 | 26 | 128 | 132 | 25 | 111 | 152 | 0.465 | Multiplex PCR |
| Hu | 2010 | China | Liver | East Asian | PB | 853 | 854 | 367 | 380 | 106 | 399 | 376 | 79 | 0.476 | TaqMan |
| Hu | 2010 | China | Cervical | East Asian | PB | 696 | 709 | 326 | 290 | 80 | 353 | 300 | 56 | 0.483 | TaqMan |
| Khaghanzadeh | 2010 | Iran | Lung | South Asian | HB | 123 | 122 | 13 | 44 | 66 | 7 | 47 | 68 | 0.763 | PCR-RFLP |
| Qi | 2010 | China | Colorectal | East Asian | HB | 124 | 407 | 60 | 60 | 4 | 183 | 179 | 45 | 0.902 | PCR-LDR |
| Kammerer | 2010 | Germany | HN | Caucasian | HB | 83 | 40 | 16 | 32 | 35 | 6 | 23 | 11 | 0.287 | RT-PCR |
| Gu | 2010 | China | Liver | East Asian | HB | 367 | 407 | 150 | 166 | 51 | 183 | 179 | 45 | 0.902 | PCR-LDR |
| Hou | 2010 | China | Gastric | East Asian | PB | 205 | 262 | 94 | 70 | 41 | 107 | 55 | 100 | 0.001 | PCR-RFLP |
| Xiong | 2010 | China | HN | East Asian | HB | 365 | 421 | 35 | 189 | 141 | 72 | 181 | 168 | 0.056 | PCR-RFLP |
| Rahimifar | 2010 | Iran | Cervical | East Asian | HB | 55 | 110 | 0 | 27 | 28 | 7 | 45 | 58 | 0.658 | PCR-RFLP |
| Pawlak | 2010 | Poland | Cervical | Caucasian | HB | 141 | 217 | 26 | 72 | 43 | 43 | 103 | 71 | 0.610 | PCR-RFLP |
| Bouwhuis | 2010 | Germany | Melanoma | Caucasian | PB | 762 | 734 | 104 | 369 | 289 | 106 | 345 | 283 | 0.956 | TaqMan |
| Xiao | 2010 | China | HN | East Asian | HB | 457 | 485 | 205 | 195 | 57 | 246 | 201 | 38 | 0.730 | PCR-RFLP |
| Dehaghani | 2009 | Iran | GTN | South Asian | NA | 83 | 84 | 7 | 34 | 42 | 2 | 37 | 45 | 0.076 | PCR-RFLP |
| Shi | 2009 | China | Pancreatic | East Asian | PB | 138 | 278 | 41 | 71 | 26 | 122 | 113 | 43 | 0.054 | PCR-RFLP |
| Lopez | 2009 | Spain | Renal | Caucasian | HB | 125 | 176 | 9 | 43 | 73 | 21 | 77 | 78 | 0.766 | TaqMan |
| Welsh | 2009 | USA | Skin | Caucasian | PB | 1581 | 819 | 214 | 707 | 660 | 148 | 353 | 318 | 0.004 | TaqMan |
| Castro | 2009 | Sweden | Cervical | Caucasian | PB | 953 | 1715 | 252 | 449 | 252 | 434 | 825 | 456 | 0.118 | Multiplex PCR |
| Dilmec | 2008 | Turkey | Colorectal | Caucasian | HB | 56 | 162 | 1 | 19 | 36 | 11 | 43 | 108 | 0.058 | PCR-RFLP |
| Sun | 2008 | China | Breast | East Asian | PB | 2097 | 2140 | 956 | 940 | 201 | 1105 | 897 | 138 | 0.053 | PCR-RFLP |
| Sun | 2008 | China | HN | East Asian | PB | 1010 | 1008 | 448 | 434 | 128 | 529 | 406 | 73 | 0.684 | PCR-RFLP |
| Sun | 2008 | China | Gastric | East Asian | PB | 530 | 530 | 235 | 235 | 60 | 282 | 209 | 39 | 0.974 | PCR-RFLP |
| Sun | 2008 | China | Lung | East Asian | PB | 2205 | 2153 | 977 | 958 | 270 | 1056 | 926 | 171 | 0.103 | PCR-RFLP |
| Suwalska | 2008 | Poland | Leukemia | Caucasian | HB | 170 | 224 | 30 | 84 | 56 | 47 | 106 | 71 | 0.524 | SNaPshot |
| Mahajan | 2008 | Poland | Gastric | Caucasian | PB | 301 | 411 | 59 | 153 | 89 | 70 | 189 | 152 | 0.393 | TaqMan |
| Nearman | 2007 | USA | Leukemia | Caucasian | NA | 26 | 96 | 4 | 8 | 14 | 19 | 51 | 26 | 0.504 | RT-PCR |
| Cozar | 2007 | Spain | Renal | Caucasian | HB | 96 | 176 | 6 | 44 | 46 | 21 | 77 | 78 | 0.766 | PCR-RFLP |
| Wang | 2007 | China | Breast | East Asian | HB | 117 | 148 | 10 | 59 | 48 | 23 | 70 | 55 | 0.926 | PCR-RFLP |
| Hadinia | 2007 | Iran | Colorectal | South Asian | HB | 105 | 190 | 6 | 47 | 52 | 14 | 59 | 117 | 0.097 | PCR-RFLP |
| Su | 2007 | China | Cervical | East Asian | HB | 139 | 375 | 60 | 62 | 17 | 178 | 155 | 42 | 0.351 | PCR-RFLP |
| Wong | 2006 | China | HN | East Asian | HB | 118 | 147 | 48 | 58 | 12 | 58 | 64 | 25 | 0.314 | PCR-RFLP |
| Cheng | 2006 | China | Lymphoma | East Asian | HB | 62 | 250 | 34 | 26 | 2 | 119 | 102 | 29 | 0.323 | PCR-RFLP |
| Chuang | 2005 | Germany | Thymoma | Caucasian | HB | 125 | 173 | 16 | 63 | 46 | 30 | 65 | 78 | 0.015 | PCR-RFLP |
| Solerio | 2005 | Italy | Colorectal | Caucasian | HB | 132 | 238 | 13 | 43 | 76 | 19 | 91 | 128 | 0.618 | PCR-RFLP |
| Piras | 2005 | Italy | Lymphoma | Caucasian | HB | 100 | 128 | 3 | 23 | 74 | 11 | 43 | 74 | 0.199 | PCR-RFLP |
| Ghaderi | 2004 | Iran | Breast | South Asian | NA | 197 | 151 | 9 | 104 | 84 | 19 | 72 | 60 | 0.716 | PCR-RFLP |
| Monne | 2004 | Italy | Lymphoma | Caucasian | HB | 44 | 76 | 1 | 7 | 36 | 6 | 32 | 38 | 0.837 | PCR-RFLP |
| Pavkovic | 2003 | Macedonia | Lymphoma | Caucasian | HB | 130 | 100 | 16 | 53 | 61 | 10 | 39 | 51 | 0.533 | PCR-RFLP |

HB: Hospital-based; HN: Head and neck; NA: Not available; PCR: Polymerase chain reaction; PB: Population based, RFLP: Restriction fragment length polymorphism.

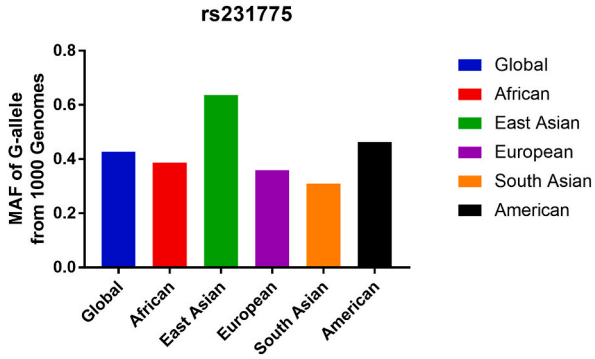


**Fig. 1.** Minor allele frequencies of CTLA-4 rs231775 A/G variation in various races.

into the "large sample" group. Stratified analyses were performed according to cancer type, race, control source, genotyping method, study quality, and sample size. Begg's funnel plot and Egger's test were used to investigate publication bias, where a *P*-value higher than 0.05 indicated no evidence of bias. All statistical analyses were performed using STATA software (Stata Company, TX, USA, version 11.0).

### *2.5. In silico and ELISA analyses of CTLA-4*

We used the National Library of Medicine (NLM) database to detect the minor allele frequencies (MAFs) in various populations (https://www.ncbi.nlm.nih.gov/snp). Additionally, we used the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) and UALCAN databases (http://ualcan.path.uab.edu/analysis.html) to assess the effect of the CTLA-4 expression profile on patient overall survival (OS) and disease-free survival (DFS). Protein-protein crosstalk of CTLA-4 in *Homo sapiens* was investigated using the online STRING server (https://string-db.org/cgi/input.pl). The transcriptomes of the BLCA samples were evaluated using the GSEA method (version 4.1.0) (http://software.broadinstitute.org/gsea/index.jsp). The CIBERSORT computational method assessed the abundance of tumor-infiltrating immune cells (TICs) in patients with BLCA. The Tumor Immune Estimation Resource (TIMER) database (https://cistrome.shinyapps.io/timer) was used to investigate the immune cell infiltration-based survival curves of patients with cancers of the urinary system. Additionally, we recruited and enrolled 200 patients with BLCA from our hospitals to conduct an ELISA evaluation of their serum CTLA-4 levels. Patients who signed a written informed consent form provided 2 mL of peripheral blood. Peripheral blood samples from participants were gathered in heparin-anticoagulant vacutainer tubes. The serum separator tube (SST) was used to solidify the specimen at room temperature for 2 h. The serum was prepared by centrifugation at 1000×*g* for 15 min and stored at minus 80° to evaluate the concentration of CTLA-4. Serum concentrations of CTLA-4 were tested following the manufacturer's instructions (https://www.cusabio.com/protocols/ELISA_Protocol.pdf. CUSABIO Co. ltd.). The study protocols were approved by the ethical committee of Changzhou Second People's Hospital.

## 3. Results

### *3.1. Characteristics of studies*

In total, 92 case-control studies involving 29,987 patients with cancer and 36,484 healthy individuals (as controls) were included in our analysis (Table 1) [11,16,21,25–97]. In the stratified analysis based on race, 64 centered on the Asian population (containing 53 East Asian and 11 South Asian studies), whereas 26 and 2 studies focused on individuals of Caucasian and African descent, respectively. In the analysis stratified by cancer type, 11 studies concentrated on colorectal and 10 on cervical cancer. Additionally, there were eight studies on both breast and lung cancers and six studies on gastric cancer, bone cancer, and lymphoma. Three studies focused on melanoma and bladder, kidney, and pancreatic cancers. Two studies were on myeloma and thyroid cancer. Tumors reported by only one study were classified into the "other cancers" group. The analysis stratified by control source included 51 hospital-based and 36 population-based studies. Five additional studies did not contain information on the control source and were classified as "not available" (NA). In the stratified analysis based on the genotyping method, 65 studies used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and 12 studies performed TaqMan assays. Four studies used the SNPscan tool, and six used PCR-ligase detection reaction (LDR). Two studies used multiplex and real time PCRs. One study used the SNaP-shot technique. The analysis stratified by research quality included 86 high-quality and six low-quality studies. Finally, the stratified analysis based on study size included 72 small-scale and 20 large-scale studies. Our search of the NLM database for information on minor allele frequencies in different populations revealed the following values: Africans, 0.388; East Asians, 0.637; Europeans, 0.359; South Asians, 0.310; Americans, 0.463; and the global population, 0.427 (Fig. 1).

**Table 2**
Stratified analysis of CTLA-4 rs231775 A/G variation on cancer susceptibility.

| Variables | N | Case/ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ |
|---|---|---|---|---|---|---|---|
| rs231775 A/G | | Control | G-allele vs. A-allele | GA vs. AA | GG vs. AA | GG + GA vs. AA | GG vs. GA + AA |
| Total | 92 | 29,987/ 36,484 | 0.95(0.90–1.00) <0.001 0.046 | 1.01(0.92–1.11) <0.001 0.827 | 0.87(0.77–0.97) <0.001 0.016 | 0.96(0.87–1.05) <0.001 0.341 | 0.88(0.82–0.94) <0.001 < 0.001 |
| **Cancer Type** | | | | | | | |
| Myeloma | 2 | 210/365 | 1.17(0.91–1.51) 0.896 0.209 | 0.91(0.53–1.56) 0.138 0.737 | 1.22(0.71–2.11) 0.420 0.478 | 1.05(0.63–1.75) 0.232 0.858 | 1.33(0.94–1.89) 0.578 0.104 |
| Bladder | 3 | 866/934 | 1.19(0.73–1.95) <0.001 0.481 | 1.23(1.01–1.51) 0.086 0.040 | 1.38(0.41–4.64) <0.001 0.603 | 1.24(0.79–1.97) 0.004 0.353 | 1.27(0.42–3.820 0.002 0.668 |
| Breast | 8 | 3785/3863 | 0.84(0.78–0.90) 0.221 < 0.001 | 0.86(0.69–1.07) 0.021 0.169 | 0.67(0.57–0.80) 0.134 < 0.001 | 0.81(0.58–1.37) 0.022 0.042 | 0.79(0.71–0.87) 0.370 < 0.001 |
| Colorectal | 11 | 3009/4208 | 1.15(0.98–1.35) <0.001 0.094 | 1.72(1.13–2.61) <0.001 0.011 | 1.24(0.81–1.90) <0.001 0.319 | 1.52(1.08–2.15) <0.001 0.017 | 0.91(0.71–1.16) <0.001 0.440 |
| Liver | 4 | 1875/2259 | 0.89(0.82–0.98) 0.151 0.018 | 0.76(0.62–0.94) 0.870 0.010 | 0.74(0.60–0.90) 0.360 0.003 | 0.75(0.61–0.91) 0.618 0.004 | 0.92(0.81–1.04) 0.164 0.187 |
| Gastric | 6 | 1971/3359 | 1.19(0.92–1.55) <0.001 0.179 | 1.42(0.96–2.12) 0.001 0.082 | 1.40(0.86–2.30) <0.001 0.179 | 1.40(0.92–2.15) <0.001 0.119 | 1.10(0.84–1.43) <0.001 0.480 |
| Cervical | 10 | 2959/4196 | 0.88(0.78–0.99) 0.023 0.028 | 0.88(0.70–1.10) 0.013 0.257 | 0.70(0.52–0.94) 0.006 0.017 | 0.83(0.66–1.03) 0.008 0.094 | 0.83(0.70–0.99) 0.039 0.043 |
| Thyroid | 2 | 488/450 | 1.50(1.22–1.85) 0.134 < 0.001 | 1.96(1.34–2.87) 0.812 0.001 | 2.42(1.48–3.95) 0.400 < 0.001 | 2.13(1.48–3.07) 0.805 < 0.001 | 1.40(1.05–1.88) 0.217 0.024 |
| Other | 6 | 3347/2706 | 0.94(0.87–1.02) 0.069 0.132 | 0.97(0.85–1.11) 0.057 0.652 | 0.86(0.65–1.14) 0.050 0.284 | 0.93(0.82–1.05) 0.096 0.216 | 0.90(0.70–1.16) 0.007 0.426 |
| Lung | 8 | 4044/4653 | 0.94(0.74–1.21) <0.001 0.643 | 0.96(0.69–1.34) <0.001 0.804 | 0.93(0.57–1.53) <0.001 0.780 | 0.92(0.62–1.35) <0.001 0.655 | 0.99(0.76–1.30) <0.001 0.964 |
| Bone | 6 | 1268/1655 | 0.82(0.63–1.05) 0.004 0.051 | 0.63(0.40–1.00) 0.001 0.051 | 0.64(0.38–1.09) 0.001 0.102 | 0.61(0.38–0.99) <0.001 0.044 | 0.81(0.69–0.95) 0.125 0.011 |
| Renal | 3 | 457/857 | 0.85(0.72–1.00) 0.143 0.056 | 0.92(0.71–1.17) 0.125 0.485 | 0.71(0.49–1.03) 0.272 0.069 | 0.85(0.67–1.08) 0.109 0.185 | 0.73(0.52–1.02) 0.485 0.062 |
| Leukemia | 3 | 282/432 | 0.84(0.64–1.09) 0.274 0.147 | 0.82(0.36–1.87) 0.034 0.629 | 0.78(0.50–1.23) 0.433 0.288 | 0.85(0.60–1.20) 0.054 0.348 | 0.78(0.54–1.11) 0.976 0.166 |
| HN | 8 | 2448/2571 | 0.79(0.69–0.91) 0.031 0.001 | 0.92(0.68–1.24) 0.004 0.577 | 0.60(0.43–0.84) 0.034 0.003 | 0.80(0.60–1.06) 0.004 0.123 | 0.69(0.53–0.88) 0.017 0.003 |
| Lymphoma | 6 | 808/1054 | 0.91(0.63–1.33) <0.001 0.625 | 0.99(0.55–1.77) <0.001 0.974 | 1.12(0.60–2.08) 0.040 0.726 | 0.96(0.53–1.76) <0.001 0.899 | 1.00(0.79–1.27) 0.264 0.985 |
| Melanoma | 3 | 1062/1067 | 1.04(0.92–1.19) 0.486 0.504 | 1.14(0.95–1.37) 0.306 0.165 | 1.00(0.76–1.33) 0.767 0.983 | 1.11(0.93–1.32) 0.349 0.233 | 0.95(0.73–1.23) 0.814 0.706 |
| Pancreatic | 3 | 1108/1855 | 0.71(0.61–0.84) 0.139 < 0.001 | 0.75(0.59–0.96) 0.444 0.022 | 0.51(0.40–0.66) 0.378 < 0.001 | 0.63(0.50–0.80) 0.421 < 0.001 | 0.87(0.84–0.90) 0.117 < 0.001 |
| **Race** | | | | | | | |
| EA | 53 | 21,808/ 26,788 | 0.96(0.90–1.03) <0.001 0.255 | 1.07(0.93–1.22) <0.002 0.338 | 0.88(0.76–1.02) <0.001 0.098 | 1.00(0.87–1.14) <0.001 0.970 | 0.88(0.81–0.95) <0.001 0.001 |
| African | 2 | 274/379 | 0.95(0.41–2.19) 0.001 0.900 | 0.93(0.28–3.10) <0.001 0.910 | 0.92(0.25–3.36) 0.027 0.904 | 0.93(0.27–3.13) <0.001 0.902 | 1.02(0.60–1.72) 0.208 0.949 |
| SA | 11 | 1540/1581 | 1.01(0.79–1.30) <0.001 0.919 | 1.04(0.79–1.38) 0.001 0.764 | 0.90(0.49–1.66) <0.001 0.745 | 1.02(0.75–1.38) <0.001 0.920 | 0.98(0.60–1.58) 0.002 0.929 |
| Caucasian | 26 | 6365/7736 | 0.89(0.80–0.98) <0.001 0.022 | 0.93(0.80–1.07) <0.001 0.291 | 0.87(0.73–1.02) 0.012 0.093 | 0.88(0.76–1.02) <0.001 0.081 | 0.87(0.84–0.90) 0.066 0.009 |
| **Source** | | | | | | | |
| HB | 51 | 9350/ 11,591 | 0.95(0.87–1.04) <0.001 0.304 | 1.03(0.88–1.20) <0.001 0.731 | 0.90(0.74–1.10) <0.001 0.301 | 0.98(0.84–1.13) <0.001 0.756 | 0.91(0.80–1.03) <0.001 0.123 |
| PB | 36 | 19,876/ 24,107 | 0.94(0.88–1.01) <0.001 0.084 | 1.00(0.88–1.12) <0.001 0.929 | 0.84(0.73–0.97) <0.001 0.020 | 0.94(0.83–1.07) <0.001 0.359 | 0.87(0.81–0.94) <0.001 < 0.001 |
| NA | 5 | 761/786 | 0.81(0.63–1.04) 0.043 0.096 | 0.90(0.72–1.13) 0.220 0.383 | 0.65(0.35–1.22) 0.031 0.182 | 0.84(0.68–1.04) 0.203 0.107 | 0.71(0.38–1.34) 0.012 0.292 |
| **Method** | | | | | | | |
| PCR-RFLP | 65 | 18,059/ 21,016 | 0.96(0.89–1.03) <0.001 0.226 | 1.03(0.92–1.16) <0.001 0.575 | 0.86(0.73–1.02) <0.001 0.078 | 0.98(0.86–1.10) <0.001 0.690 | 0.87(0.79–0.95) <0.001 0.003 |
| TaqMan | 12 | 5480/5355 | 0.88(0.77–1.00) <0.001 0.056 | 0.97(0.71–1.31) <0.001 0.819 | 0.79(0.60–1.03) <0.001 0.076 | 0.87(0.67–1.13) <0.001 0.301 | 0.81(0.69–0.95) 0.007 0.010 |
| SNPscan | 4 | 2561/4717 | 0.94(0.87–1.00) 0.106 0.076 | 0.87(0.74–1.04) 0.356 0.117 | 0.84(0.71–1.00) 0.146 0.046 | 0.86(0.73–1.00) 0.233 0.062 | 0.94(0.85–1.04) 0.146 0.224 |
| PCR-LDR | 6 | 2369/3033 | 1.01(0.93–1.10) 0.130 0.800 | 1.10(0.76–1.60) 0.008 0.603 | 1.09(0.77–1.55) 0.015 0.619 | 1.10(0.77–1.56) 0.008 0.606 | 1.02(0.92–1.14) 0.544 0.715 |
| M-PCR | 2 | 1239/2003 | 1.05(0.95–1.16) 0.312 0.348 | 1.06(0.89–1.25) 0.138 0.516 | 1.07(0.87–1.31) 0.686 0.535 | 1.07(0.91–1.25) 0.176 0.405 | 1.06(0.89–1.26) 0.978 0.506 |
| RT-PCR | 2 | 109/136 | 0.67(0.44–1.01) 0.298 0.056 | 0.37(0.19–0.71) 0.544 0.003 | 0.58(0.25–1.33) 0.382 0.199 | 0.42(0.23–0.77) 0.427 0.005 | 1.04(0.49–2.20) 0.445 0.923 |

(*continued on next page*)

**Table 2** (*continued*)

| Variables | N | Case/ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ | OR(95%CI) $P_{heter}$ $P$ |
|---|---|---|---|---|---|---|---|
| rs231775 A/G | | Control | G-allele vs. A-allele | GA vs. AA | GG vs. AA | GG + GA vs. AA | GG vs. GA + AA |
| SNaPshot | 1 | 170/224 | 0.91(0.69–1.21) - 0.521 | 1.00(0.64–1.58) - 0.984 | 0.81(0.46–1.44) - 0.472 | 0.95(0.62–1.45) - 0.793 | 0.81(0.49–1.34) - 0.409 |
| **Quality** | | | | | | | |
| High | 86 | 27,391/ 34,505 | 0.94(0.89–0.99) <0.001 0.023 | 0.98(0.90–1.06) <0.001 0.582 | 0.85(0.76–0.95) <0.001 0.004 | 0.93(0.85–1.02) <0.001 0.129 | 0.89(0.83–0.95) <0.001 < 0.001 |
| Low | 6 | 2596/1979 | 0.98(0.66–1.45) <0.001 0.902 | 1.73(0.82–3.66) <0.001 0.150 | 1.05(0.50–2.21) <0.001 0.903 | 1.36(0.71–2.58) <0.001 0.353 | 0.77(0.50–1.20) <0.001 0.255 |
| **Size** | | | | | | | |
| Small | 72 | 12,836/ 16,420 | 0.96(0.89–1.04) <0.001 0.308 | 1.07(0.95–1.21) <0.001 0.289 | 0.90(0.76–1.06) <0.001 0.215 | 1.01(0.89–1.14) <0.001 0.935 | 0.88(0.79–0.97) <0.001 0.012 |
| Large | 20 | 17,151/ 20,064 | 0.90(0.84–0.96) <0.001 0.002 | 0.85(0.77–0.95) 0.001 0.003 | 0.78(0.67–0.90) <0.001 0.001 | 0.82(0.72–0.93) <0.001 0.001 | 0.89(0.82–0.96) <0.001 0.003 |

HB: Hospital based; EA: East Asian; SA: South Asian; HN: Head and neck cancer; PCR: polymerase chain reaction; M-PCR: Multiplex PCR; RT: Real time; N: Number of included studies; NA: Not available; PB: Population based. $P_{heter}$: $P$ value of heterogeneity test.

### 3.2. Main results

Overall, the allele contrast, homozygote contrast, and recessive models revealed that the rs231775 A/G polymorphism was associated with a decreased cancer risk. Compared with the individuals carrying the A-allele, those carrying the G-allele were associated with a reduced cancer risk (OR = 0.95, 95% CI = 0.90–1.00, $P = 0.046$, Table 2). In the stratified analysis based on cancer type, seven studies concentrated on urinary system cancer [BLCA, PRAD, and kidney renal clear cell carcinoma (KIRC)]. We revealed that individuals with the AA genotype had a 23% lower risk of developing BLCA than those with the GG genotype (OR = 1.23, 95% CI = 1.01–1.51, $P = 0.040$, Fig. 2). By contrast, no significant association was observed for patients with PRAD or KIRC. Moreover, we observed that individuals with the GG genotype had a 33% lower risk of developing breast cancer than those with the AA genotype (OR = 0.67, 95% CI = 0.57–0.80, $P < 0.001$). The allele contrast model for liver cancer revealed that the polymorphism was associated

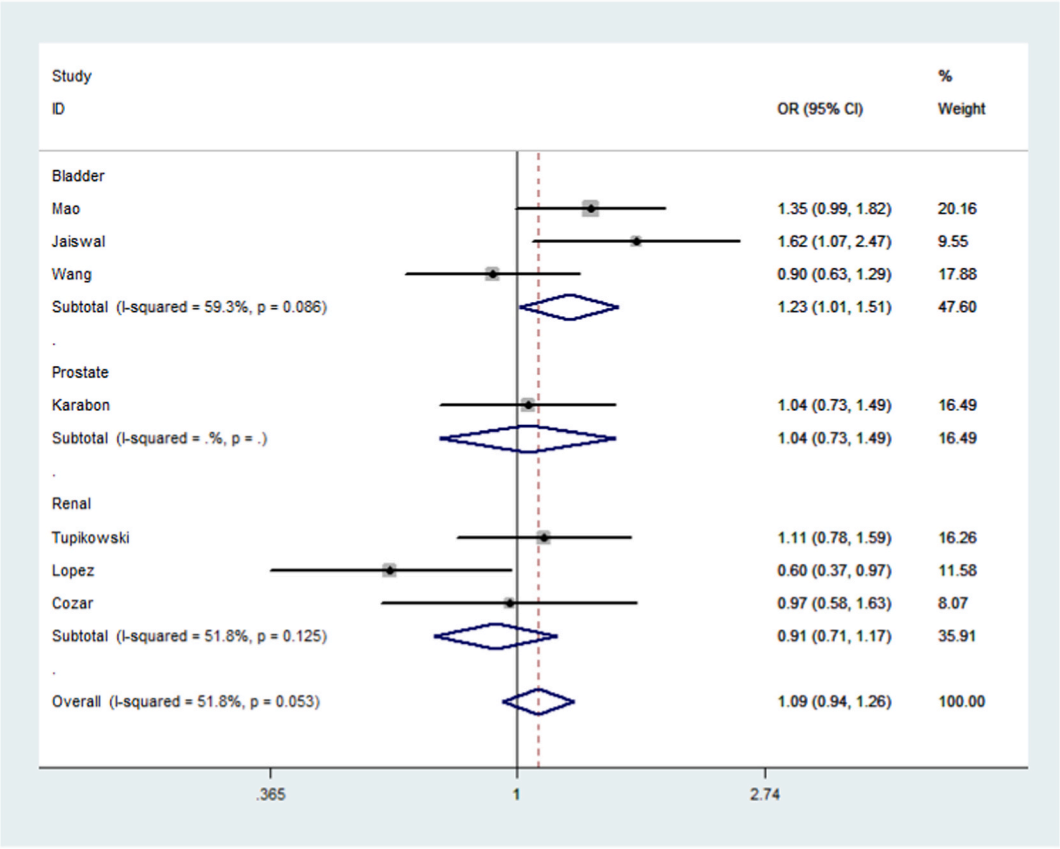


**Fig. 2.** Forest plot of the association between CTLA-4 rs231775 A/G variant and risk of urinary cancer.

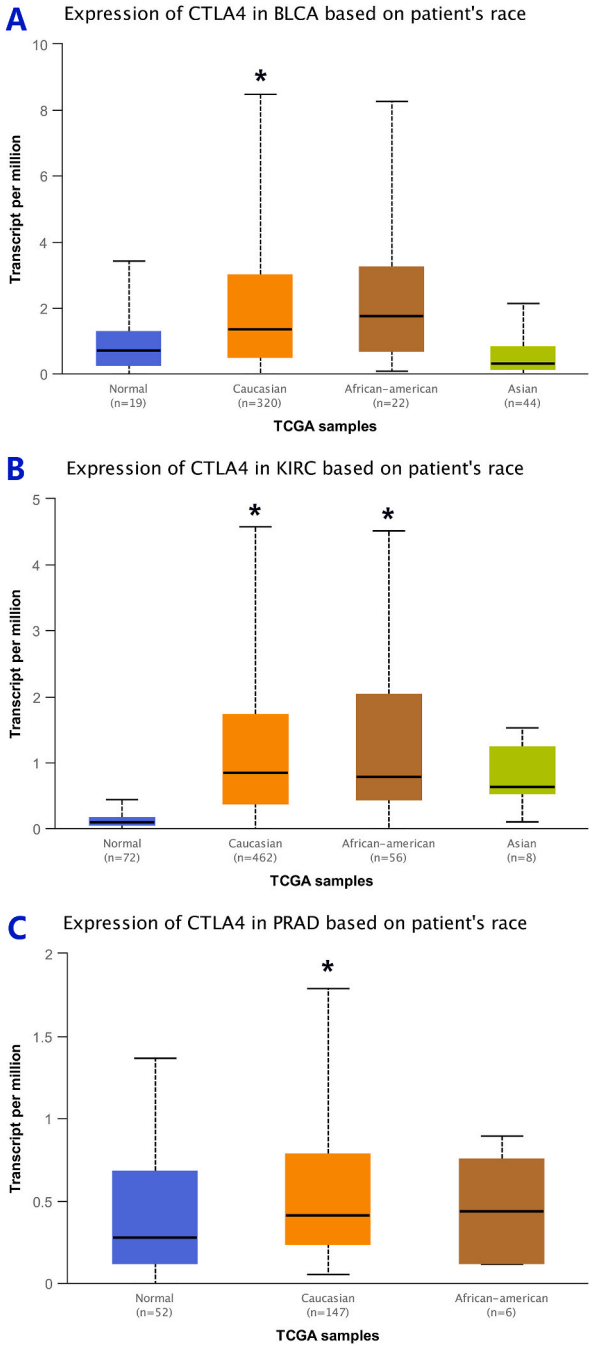


**Fig. 3.** *In silico* analysis of CTLA-4 expression in bladder cancer (BLCA, Figure A), kidney renal clear cell carcinoma (KIRC, Figure B), and prostate cancer (PRAD, Figure C) based on patients' race. The expression of CTLA-4 was up-regulated in BLCA patients with Caucasian descent, as compared with normal control. For KIRC patients, the expression of CTLA-4 was augmented in Caucasian and African-American descendants. For PRAD, CTLA-4 expression was enhanced in patients with Caucasian descent.

with diminished cancer risk (OR = 0.89, 95% CI = 0.82–0.98, $P = 0.018$). We observed similar result in heterozygote contrast (OR = 0.76, 95% CI = 0.62–0.94, $P = 0.010$), homozygous comparison (OR = 0.74, 95% CI = 0.60–0.90, $P = 0.003$), and dominant model (OR = 0.75, 95% CI = 0.61–0.91, $P = 0.004$). Similarly, the gene variant correlated with reduced risks of cervical, bone, pancreatic, and head and neck cancer development (Table 2). Moreover, it was observed to be associated with an elevated risk of thyroid cancer according to the allelic contrast (OR = 1.50, 95% CI = 1.22–1.85, $P < 0.001$), heterozygote contrast (OR = 1.96, 95% CI = 1.34–2.87, $P = 0.001$), homozygous comparison (OR = 2.42, 95% CI = 1.48–3.95, $P < 0.001$), dominant model (OR = 2.13, 95% CI = 1.48–3.07, $P < 0.001$) and recessive model (OR = 1.40, 95% CI = 1.05–1.88, $P = 0.024$). In the analysis stratified by race, East Asians with the GG

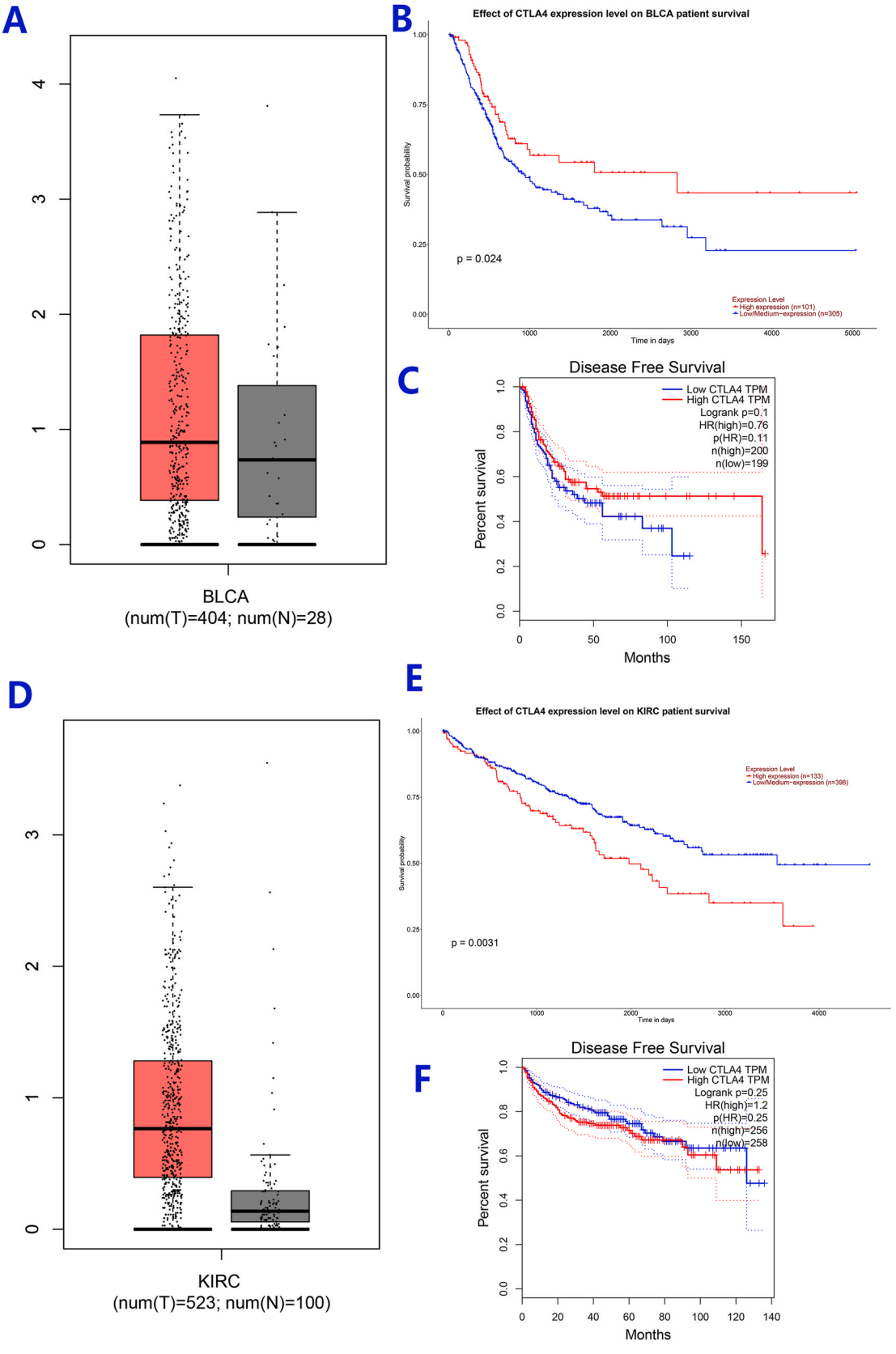
A
BLCA
(num(T)=404; num(N)=28)
B
Effect of CTLA4 expression level on BLCA patient survival
Survival probability
p = 0.024
Expression Level
High expression (n=101)
Low/Medium−expression (n=305)
Time in days
C
Disease Free Survival
Low CTLA4 TPM
High CTLA4 TPM
Logrank p=0.1
HR(high)=0.76
p(HR)=0.11
n(high)=200
n(low)=199
Percent survival
Months
D
KIRC
(num(T)=523; num(N)=100)
E
Effect of CTLA4 expression level on KIRC patient survival
Expression Level
High expression (n=133)
Low/Medium−expression (n=398)
Survival probability
p = 0.0031
Time in days
F
Disease Free Survival
Low CTLA4 TPM
High CTLA4 TPM
Logrank p=0.25
HR(high)=1.2
p(HR)=0.25
n(high)=256
n(low)=258
Percent survival
Months

**Fig. 4.** Effect of CTLA-4 expression on survival of urinary cancer patients. The expression of CTLA-4 was increased in BLCA ($P < 0.05$, Figure A) and KIRC patients ($P < 0.05$, Figure D). BLCA patients with low expression of CTLA-4 may have a shorter overall survival (OS) time than high CTLA-4 expression group ($P < 0.05$, Figure B). Effect of CTLA-4 level on BLCA patients' disease-free survival (DFS) time was shown in Figure C. No significant difference on the DFS was revealed ($P > 0.05$). KIRC patients with high expression of CTLA-4 may have a shorter OS time than low expression group (Figure E). No significant difference was revealed for PRAD (Figure G, H, and I).

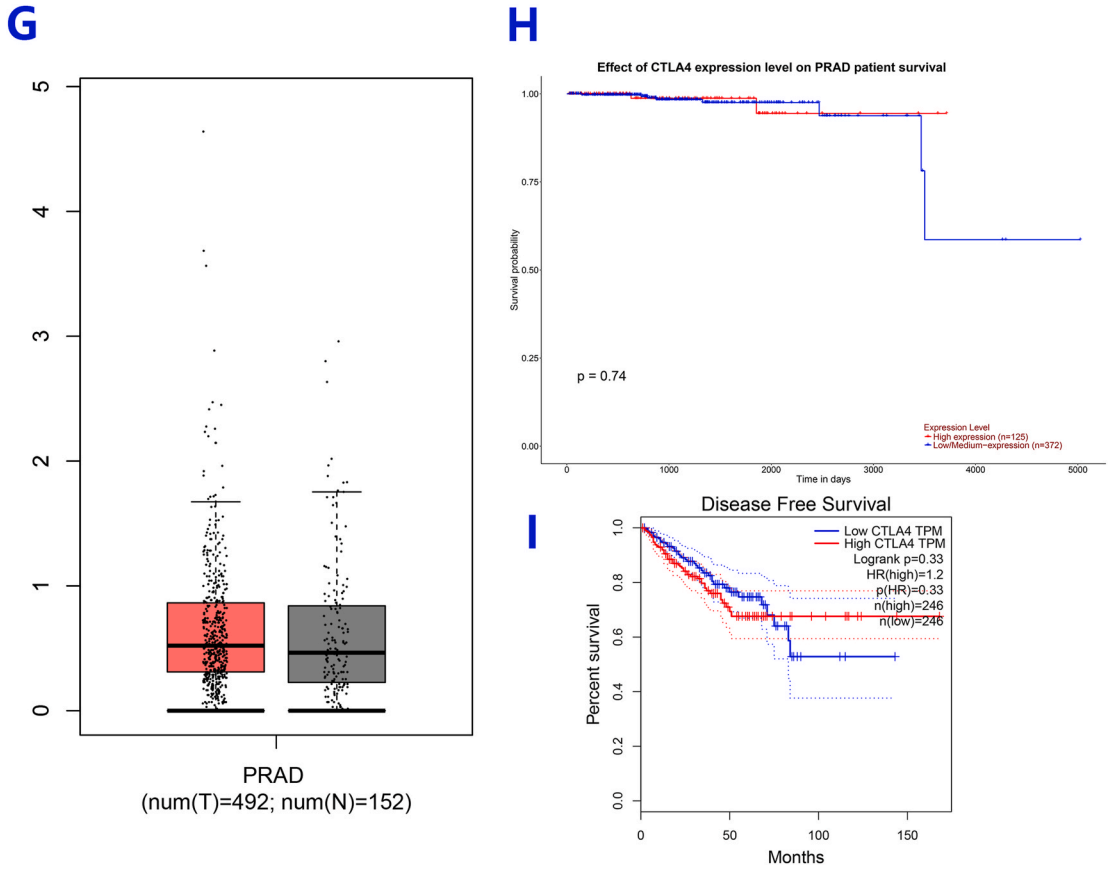


**Fig. 4.** (*continued*).

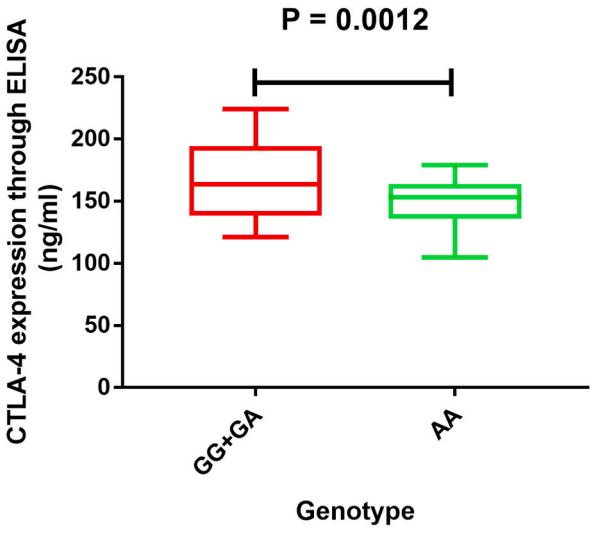


**Fig. 5.** Analysis of serum CTLA-4 levels in BLCA subjects by ELISA. Compared with those with GG + GA genotype, the expression of CTLA-4 was mitigated in BLCA participants with AA genotype ($P < 0.05$).

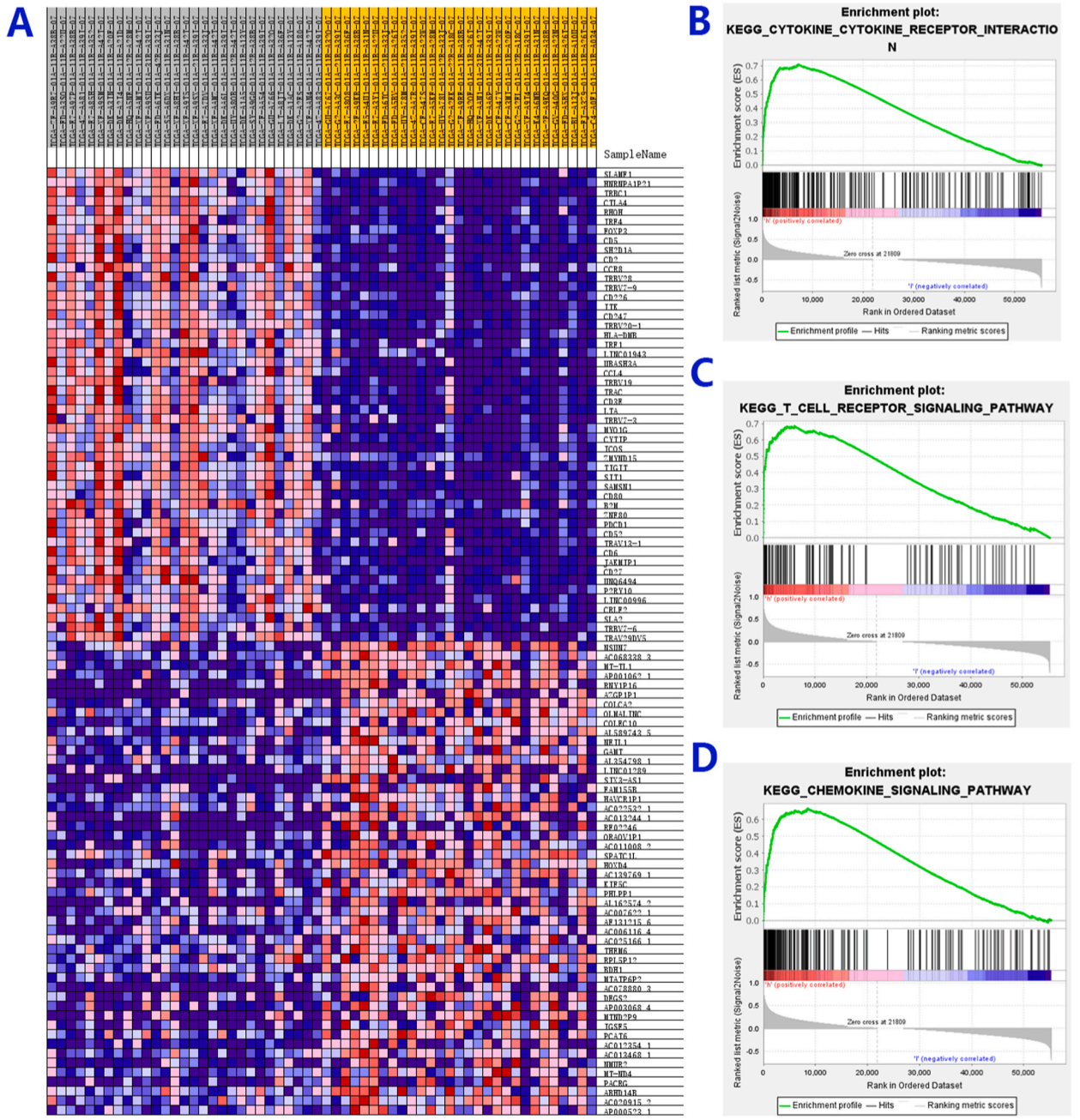


**Fig. 6.** Gene set enrichment analysis (GSEA) of CTLA-4 expression samples. The enrichment heat map was described in Figure A. Signaling pathways, including cytokine-cytokine receptor interaction (Figure B), T cell receptor signaling pathway (Figure C), chemokine signaling pathway (Figure D) were associated with high expression of CTLA-4.

genotype had a 12% lower risk of developing cancer than those with the GA + AA genotype. Similar results were observed for the Caucasian population (allelic contrast, OR = 0.89, 95% CI = 0.80–0.98, $P = 0.022$; recessive model, OR = 0.87, 95% CI = 0.84–0.90, $P = 0.009$). By contrast, no significant association was observed for individuals of African or South Asian descent. In the analysis stratified by genotyping method, the gene variant was associated with decreased cancer risk, especially in studies using PCR-RFLP, TaqMan, and RT-PCR methods. In the analysis stratified by control source, the homozygous comparison model revealed that the polymorphism was correlated with a reduced risk of cancer in population-based studies (OR = 0.84, 95% CI = 0.73–0.97, $P = 0.020$) and the recessive model (OR = 0.87, 95% CI = 0.81–0.94, $P < 0.001$). The analysis stratified based on research quality revealed a positive association between the polymorphism and cancer risk in high-quality studies. The analysis based on sample size revealed that individuals carrying the G-allele had a 10% lower risk of developing cancer than those carrying the A-allele in studies with a large-

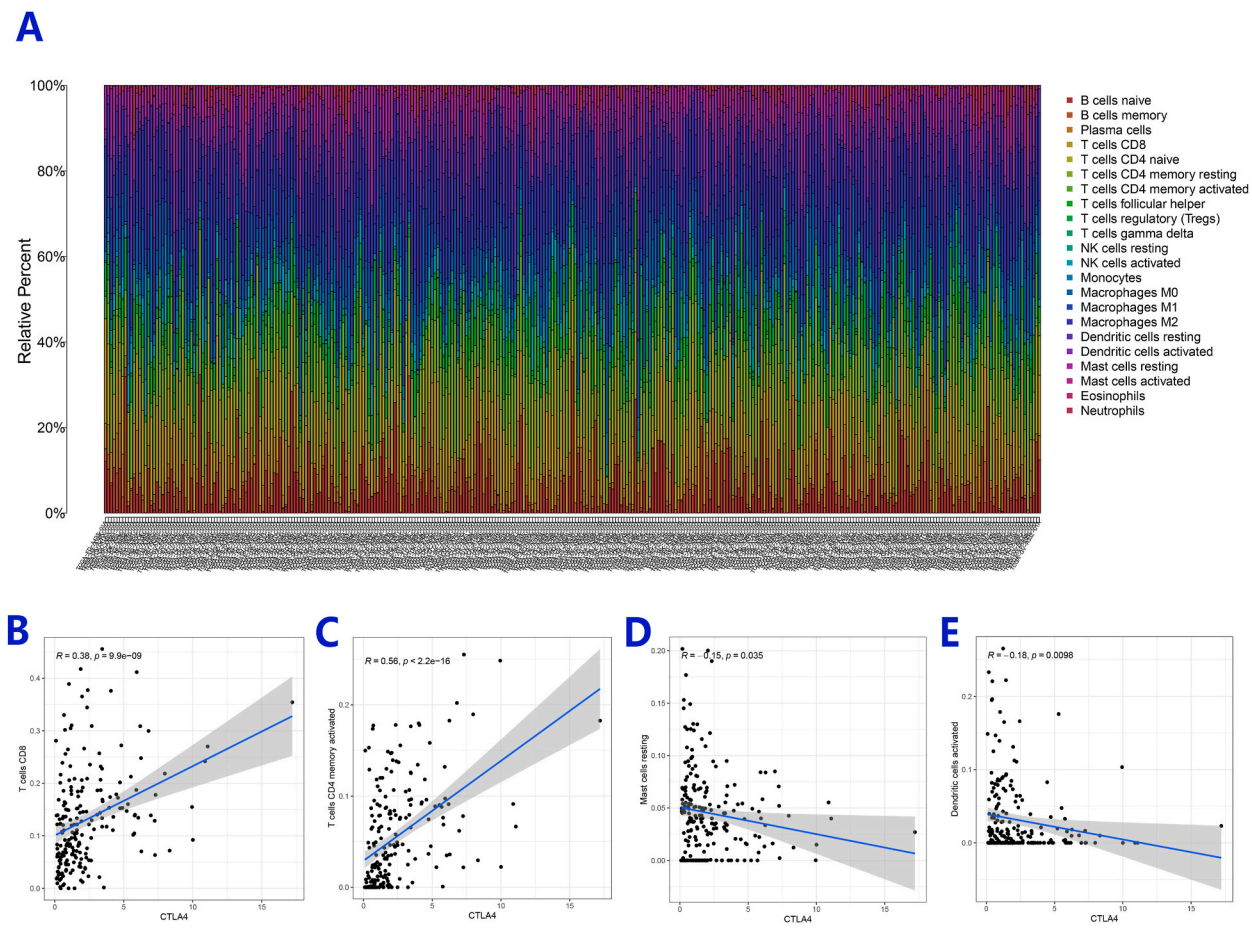


**Fig. 7.** Association between the tumor-infiltrating immune cell (TIC) abundance profile and CTLA-4 expression in samples with BLCA. Proportion of 22 immune cell lines in high and low CTLA-4 expression groups was shown in Figure A. $CD8^+$ T cells (Figure B) and CD4 memory activated T cells (Figure C) were positively correlated with expression of CTLA-4. Resting mast cells (Figure D) and activated dendritic cells (Figure E) were negatively associated with the CTLA-4 expression.

sample size (95% CI = 0.84–0.96, $P = 0.002$).

### 3.3. *In silico and ELISA analyses of CTLA-4*

We used *in silico* tools to investigate the expression of CTLA-4 in patients with BLCA, PRAD, and KIRC. As shown in Fig. 3, CTLA-4 expression was augmented in patients with BLCA of Caucasian descent compared with that in healthy individuals (Fig. 3A). Among patients with KIRC, the expression of CTLA-4 was increased in individuals of Caucasian and African-American descent (Fig. 3B). Patients with PRAD of Caucasian descent had enhanced CTLA-4 expression (Fig. 3C). Our evaluation of the GEPIA and UALCAN databases to evaluate the effect of CTLA-4 expression on the survival of patients with cancers of the urinary system revealed that the protein expression level was increased in patients with BLCA ($P < 0.05$, Fig. 4A) and KIRC ($P < 0.05$, Fig. 4D). Moreover, patients with BLCA having low-level CTLA-4 expression may have a shorter OS time than those with high CTLA-4 levels ($P < 0.05$, Fig. 4B). The effects of high/low CTLA-4 levels on the DFS time of patients' with BLCA was shown in Fig. 4C; no significant difference was evident between the two groups ($P > 0.05$, Fig. 4F). Patients with KIRC expressing high levels of CTLA-4 may have a shorter OS time than those in low-expression group (Fig. 4E). For patients with PRAD, the CTLA expression level had no notable effect on either OS or DFS ($P > 0.05$, Fig. 4H and I).

Our ELISA analysis of the serum CTLA-4 levels in patients with BLCA recruited from our hospitals revealed that the individuals carrying the AA genotype had lower levels of protein than those with the GG + GA genotype ($P < 0.05$, Fig. 5). Concerning identifying signaling pathways potentially related to CTLA-4 expression, the enrichment heat map generated with the GSEA tool was represented in Fig. 6A. The cytokine-cytokine receptor interactions (Fig. 6B), T-cell receptor signaling (Fig. 6C), and chemokine signaling, (Fig. 6D) were specifically found to be associated with high CTLA-4 expression. Furthermore, we used the CIBERSORT technique to evaluate the abundance profile of TICs in the BLCA samples (Fig. 7A). The proportions of $CD8^+$ T cells (R = 0.38, $P < 0.05$, Fig. 7B) and $CD4^+$ memory-activated T cells (R = 0.56, $P < 0.05$, Fig. 7C) correlated positively with CTLA-4 expression. By contrast, CTLA-4 expression

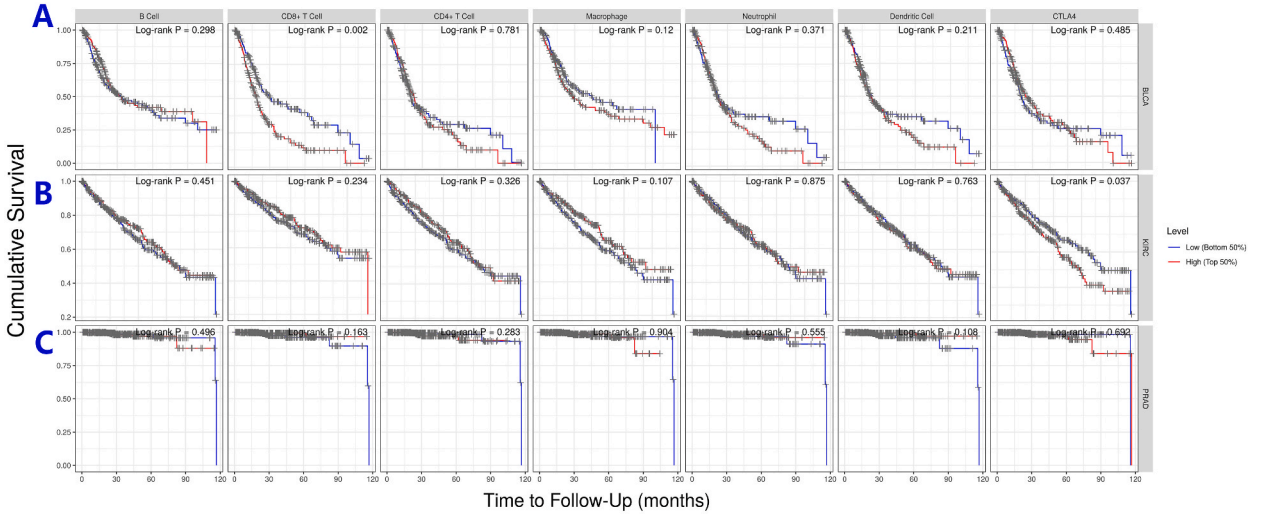


**Fig. 8.** Effects of CTLA-4 expression and immune cell infiltration on survival curve of patients with urinary cancer. $CD8^+$ T cell infiltration were significantly associated with the clinical outcome of BLCA patients (Fig. 8A). For KIRC, individuals with high expression of CTLA-4 may have a shorter survival time than those with low CTLA-4 expression groups (Fig. 8B). This result was consistent with that in Fig. 4E. For PRAD, no positive result was revealed (Fig. 8C).

correlated negatively with the proportion of resting mast cells ($R = -0.15$, $P < 0.05$, Fig. 7D) and activated dendritic cells ($R = -0.18$, $P < 0.05$, Fig. 7E). Our TIMER database investigation to explore the effects of CTLA-4 expression and immune cell infiltration on the survival of patients with cancers of the urinary system showed that $CD8^+$ T cell infiltration was significantly associated with the clinical outcomes in patients with BLCA ($P < 0.05$, Fig. 8A). In the case of KIRC, patients with high CTLA-4 expression may have shorter survival times than those with low expression of the protein ($P < 0.05$, Fig. 8B). No positive results were revealed for patients with PRAD ($P > 0.05$, Fig. 8C). Additionally, our analysis of the gene-gene correlations of CTLA-4 based on the UALCAN database identified more than 24 genes that interact with CTLA-4 in BLCA (Fig. 9A), the top three being those encoding T-cell surface antigen CD2 (*CD2*, Fig. 9B), inducible T cell costimulator (*ICOS*, Fig. 9C), and T-cell surface glycoprotein CD3 zeta chain (*CD247*, Fig. 9D). The molecules that crosstalk with CTLA-4 were investigated using the STRING tool (Fig. 10A), which identified the ICOS ligand, ENSP00000483732 as the most closely related to the protein (Fig. 10B).

### 3.4. Publication bias

We used Begg's funnel plots to explore potential publication bias in CTLA-4 rs231775 A/G polymorphism studies. No obvious asymmetry was observed in the plots (Fig. 11A), indicating a lack of bias. This was confirmed using Egger's tests. Fig. 11B suggested that the publication bias of each study addressing this polymorphism in cancer was negligible and may have had little impact on the final results ($P > 0.05$).

## 4. Discussion

CTLA-4, which belongs to the immunoglobulin superfamily, is involved in immune disorders associated with various cancers [12]. It also acts in the early stage of immune response and can weaken the anti-tumor immune activity and promote the growth and metastasis of cancer cells. Previous study has shown that the expression of CTLA-4 can be down-regulated by capecitabine, suggesting that targeting CTLA-4 can play a potential therapeutic role in colorectal cancer [24]. CTLA-4 is important for allowing carcinomas to evade host immune surveillance [13]. SNPs can affect gene expression and are associated with cancer risk in individuals of various racial populations [38,78,82]. The 49A allele leads to better mRNA efficiency, resulting in higher CTLA-4 expression than that yielded by the 49G allele [11]. Numerous studies have investigated the effect of the CTLA-4 rs231775 A/G polymorphism on cancer risk; however, their results remain contradictory. Gokhale et al. revealed that the rs231775 AA genotype could increase the risk of cervical cancer [97]. However, this finding could not be confirmed by another case-control study [89]. In this present study, we comprehensively analyzed 92 case-control studies involving 29,987 patients with cancer and 36,484 healthy individuals (controls) to better explain the associations of the CTLA-4 rs231775 variant with cancer risk and different racial population. Moreover, we used GSEA, *in silico* tools, and ELISA to explore the effects of CTLA-4 expression on patients with BLCA.

Our pooled analysis based on the allele contrast, homozygote contrast, and recessive models revealed that the rs231775 A/G polymorphism was associated with decreased cancer risk. However, the mutation appears to play different roles in various cancers. In the stratified analysis based on cancer type, we observed that the gene variant correlated with a decreased risk of liver, breast, cervical, bone, and pancreatic cancers. Our findings are consistent with those of a previous meta-analysis [18]. Furthermore, we observed that

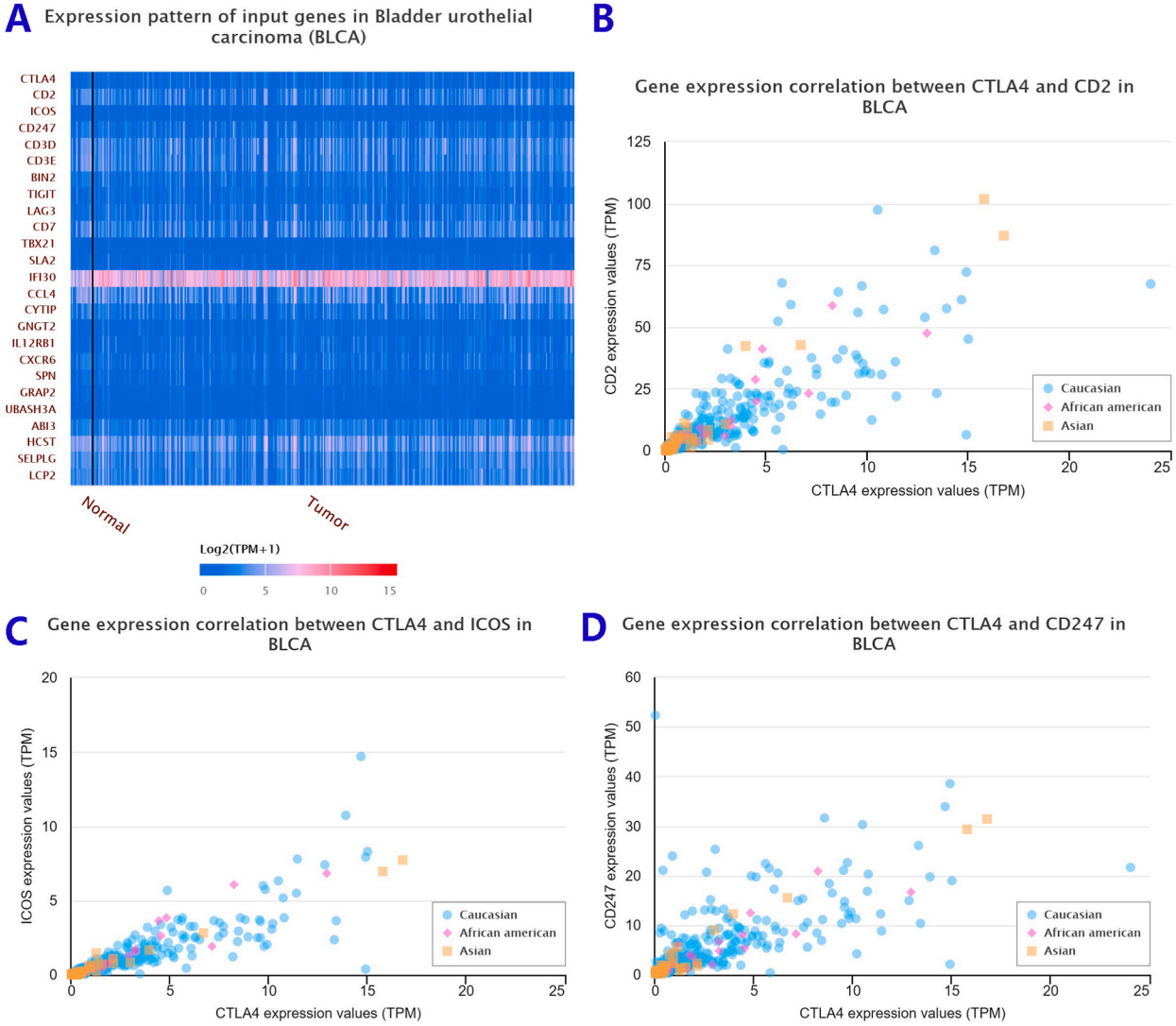


**Fig. 9.** Gene-gene correlation of CTLA-4 in BLCA patients. Expression pattern of input genes in BLCA was described in Figure A. More than 20 genes were involved in the correlation with CTLA-4. The most related gene with CTLA-4 in BLCA were CD2 (CD2 molecule, also known as T11, Figure B), inducible T cell costimulator (ICOS, Figure C), and CD247 (CD247 molecule, also known as CD3-ZETA, Figure D).

the rs231775 A/G polymorphism was associated with an increased risk of BLCA and thyroid cancers. In the analysis stratified by race, we observed that East Asians with the GG genotype had a 12% lower risk of developing cancer than those with the GA + AA genotype. Similar results were observed for the Caucasian populations. By contrast, a meta-analysis conducted by Wang et al. [98] concluded that no significant risk associations existed. This may be because the sample size of their meta-analysis was relatively small. Furthermore, we observed that the association of the CTLA-4 rs231775 A/G polymorphism with cancer risk was evident in high-quality studies with large sample size. Additionally, we used *in silico* tools to investigate the expression of CTLA-4 in patients with urinary system cancers. The expression of CTLA-4 was higher in patients with BLCA of Caucasian descent than in the individuals in the control group. Similarly, CTLA-4 expression was enhanced in patients with PRAD of Caucasian descent and patients with KIRC of Caucasian and African-American descent. Patients with BLCA expressing low levels of CTLA-4 may have a shorter OS time than those expressing high protein levels. Moreover, among our hospital patients with BLCA, those with the AA genotype had lower levels of CTLA-4 protein than the patients with the GG + GA genotype.

Although this study has the advantage of a large sample size, it has certain limitations that must be considered. First, studies on the association between the CTLA-4 rs231775 A/G polymorphism and urinary system cancers are limited, our sample size for determining this particular relationship was small. As observed in the analysis stratified by research quality, a positive association between the gene variant and cancer risk was revealed in the high-quality studies. Thus, more high-quality studies are warranted to explore the underlying correlation between the CTLA-4 rs231775 A/G mutation and the risk of developing urinary system cancer. Second, the potential heterogeneity of the included studies was not verified. The analysis stratified by genotyping methods suggested that the gene

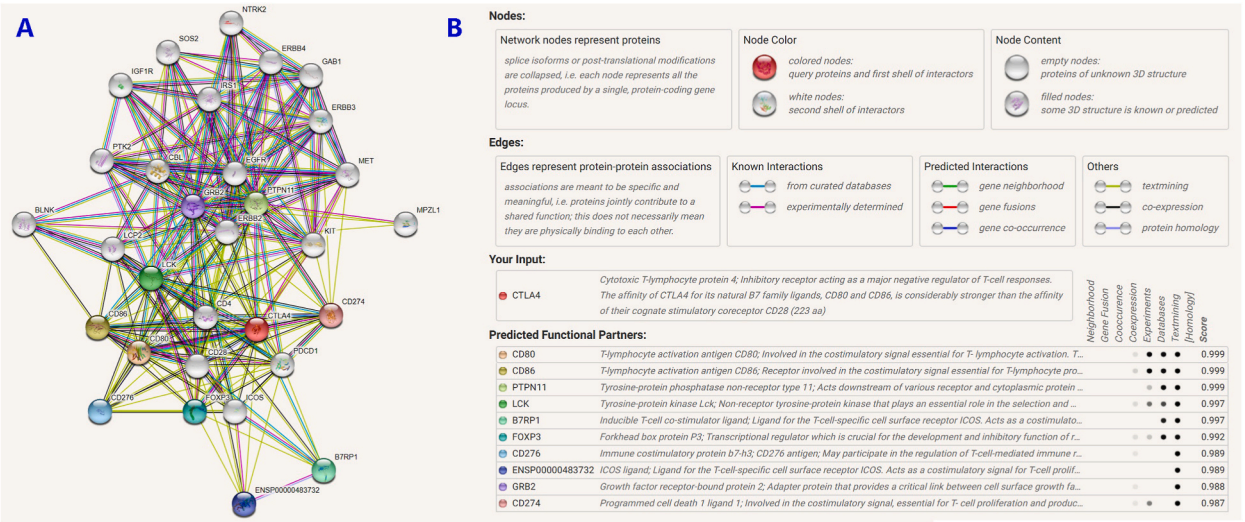


**Fig. 10.** The crosstalk of CTLA-4 protein investigated by STRING tools. More than 30 proteins contributor to the correlation with CTLA-4 protein (Figure A). The 10 most related proteins are as follows: T-lymphocyte activation antigen CD80, T-lymphocyte activation antigen CD86, Tyrosine-protein phosphatase non-receptor type 11 (PTPN11), Tyrosine-protein kinase Lck (LCK), Inducible T-cell co-stimulator ligand (B7RP1), Forkhead box protein P3 (FOXP3), Immune costimulatory protein b7-h3 (CD276), inducible T cell costimulator ligand (ENSP00000483732), Growth factor receptor-bound protein 2 (GRB2), Programmed cell death 1 ligand 1 (CD274) (Figure B).

variant was associated with decreased cancer risk, a fact especially established in studies that used the PCR-RFLP, TaqMan, and RT-PCR techniques. Therefore, the effect of the rs231775 A/G polymorphism on cancer risk may be influenced by different genotyping methods. Third, as the data on gene-environment interactions were not unified among the included studies, it was difficult to demonstrate an association between the polymorphism and patients with cancer having different lifestyle factors, including sex, diet, and a history of drinking and smoking. Fourth, the ELISA analysis revealed that the expression of CTLA-4 was reduced in patients with BLCA carrying the AA genotype. More functional studies are required to explore whether this CTLA-4 gene mutation affects protein expression in BLCA. Fifth, the health status of the control group only indicates that of the participants at a specific point in time. Some controls may also develop cancers over time. In the present analysis, we did not follow up the health status of controls over a long period of time. Moreover, in the ELISA analyses, it is reasonable to assess the expression levels of CTLA-4 in control group; however, we did not obtain the ethical approval to take the specimens from healthy individuals. GSEA revealed that signaling pathways, such as those for cytokine-cytokine receptor interaction, T cell receptor signaling, and chemokine signaling, were associated with the expression of CTLA-4 in BLCA. As identified by the *in silico* tools, more than 24 genes interacted with CTLA-4 in BLCA. Furthermore, over 30 proteins were involved in crosstalk with the CTLA-4 protein. The specific interactions between CTLA-4 and the indicated pathways require further verification through functional studies.

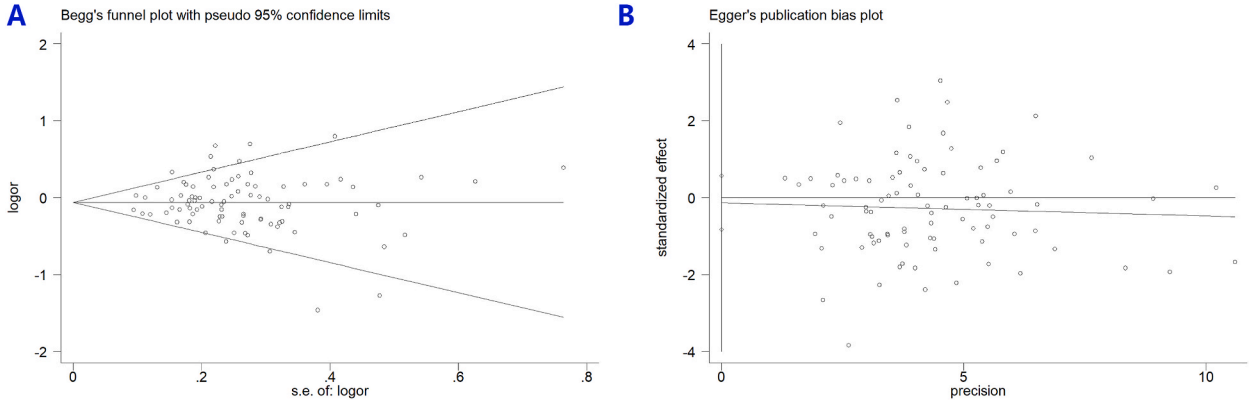


**Fig. 11.** Publication bias of the present analysis measured by Begg's and Egger's test. No evidence of publication bias was revealed by Begg's plot (Figure A) and Egger's tests (Figure B).

## 5. Conclusion

In summary, we comprehensively analyzed all eligible studies to explore the association between the CTLA-4 rs231775 variant and the risk of cancer. We revealed that the rs231775 A/G polymorphism could impact the predisposition to certain cancer types, particularly BLCA. *In silico* analysis indicated that CTLA-4 expression was augmented in patients with BLCA; however, this was mitigated in the individuals carrying the AA genotype. Several signaling pathways, including those for cytokine-cytokine receptor interactions and T cell receptor signaling, were observed to be associated with CTLA-4 expression. The detection of CTLA-4 gene polymorphisms and the expression of CTLA-4 in high-risk groups may provide guidance for the detection and prognosis of BLCA.

## Data accessibility

The original data presented in the study can be acquired from the article. Further inquiries are available from contact to the corresponding authors.

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## Ethics approval and consent to participate

The current study has been approved by Ethics Committee of Changzhou Second People's Hospital of Nanjing Medical University (Number: [2020]KY223-01).

## Additional information

No additional information is available for this paper.

## CRediT authorship contribution statement

**Haiyan Pan:** Data curation. **Zebin Shi:** Writing – review & editing, Visualization. **Lei Gao:** Resources, Methodology. **Li Zhang:** Resources, Investigation. **Shuzhang Wei:** Supervision, Software. **Yin Chen:** Software, Formal analysis. **Chao Lu:** Formal analysis, Data curation. **Jianzhong Wang:** Validation, Conceptualization. **Li Zuo:** Project administration, Conceptualization. **Lifeng Zhang:** Writing – review & editing, Writing – original draft, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

## Abbreviations

| | |
|---|---|
| BLCA | bladder cancer |
| CTLA-4 | cytotoxic T-lymphocyte associated antigen-4 |
| DFS | disease-free survival |
| ELISA | enzyme linked immunosorbent assay |
| GSEA | Gene Set Enrichment Analysis |
| EA | East Asian |
| HN | Head and neck cancer |
| ICOS | inducible T cell costimulator |
| HWE | Hardy-Weinberg equilibrium |
| LDR | ligase detection reaction |
| MAF | minor allele frequency |
| NLM | National Library of Medicine |
| PCR | polymerase chain reaction |
| M-PCR | Multiplex PCR |
| RT | Real time |

| | |
|---|---|
| NA | Not available |
| PB | Population based |
| RFLP | Restriction fragment length polymorphism |
| HB | Hospital based |
| SA | South Asian |
| OS | overall survival |
| TIC | tumor-infiltrating immune cell |

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