Inferring tumour purity and stromal and immune cell admixture from expression data

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Infiltrating stromal and immune cells form the major fraction of normal cells in tumour tissue and not only perturb the tumour signal in molecular studies but also have an important role in cancer biology. Here we describe ‘Estimation of STromal and Immune cells in MAInflictant Tumours using Expression data’ (ESTIMATE)—a method that uses gene expression signatures to infer the fraction of stromal and immune cells in tumour samples. ESTIMATE scores correlate with DNA copy number-based tumour purity across samples from 11 different tumour types, profiled on Agilent, Affymetrix platforms or based on RNA sequencing and available through The Cancer Genome Atlas. The prediction accuracy is further corroborated using 3,809 transcriptional profiles available elsewhere in the public domain. The ESTIMATE method allows consideration of tumour-associated normal cells in genomic and transcriptomic studies. An R-library is available on https://sourceforge.net/projects/estimateproject/.

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Malignant solid tumour tissues consist of not only tumour cells but also tumour-associated normal epithelial and stromal cells, immune cells and vascular cells. Stromal cells are thought to have important roles in tumour growth, disease progression and drug resistance. Infiltrating immune cells act in a context-dependent manner, and whereas antitumor effects of infiltrating T-lymphocytes have been observed in ovarian cancer, associations with tumour growth, invasion and metastasis were described in colorectal cancer. The comprehensive understanding of tumour-associated normal cells in tumour tissues may provide important insights into tumour biology and aid in the development of robust prognostic and predictive models.

Gene expression profiling of cancer has resulted in the identification of molecular subtypes and the development of models for prediction prognosis and has enriched our knowledge of the molecular pathways of tumorigenesis. Increasing evidence suggests that the infiltration of tumour-associated normal cells influences the analysis of clinical tumour samples by genomic approaches, such as gene expression profiles or copy number data, and biological interpretation of the results requires considerable attention to sample heterogeneity. Several methods have been proposed to estimate the fraction of tumour cells in clinical tumour samples by using DNA copy number array data or by using next-generation sequencing data. DNA copy number-based estimation of tumour purity is rapidly gaining traction in predicting the purity of tumour samples; however, such methods are limited to samples with available copy number profiles. Previous studies have attempted to deconvolve gene expression data into gene expression profiles from their constituent cellular fractions, whereas others have focused on deconvolution of microarray data obtained from normal tissue into cell-type-specific profiles, by calculating enrichment scores. These methods take advantage of the differences in transcriptome properties of distinct cell types.

Here we present a new algorithm that takes advantage of the unique properties of the transcriptional profiles of cancer samples to infer tumour cellularity as well as the different infiltrating normal cells, called ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumour tissues using Expression data). We focus on stromal and immune cells that form the major non-tumour constituents of tumour samples and identify specific signatures related to the infiltration of stromal and immune cells in tumour tissues. By performing single-sample gene set-enrichment analysis (ssGSEA), we calculate stromal and immune scores to predict the level of infiltrating stromal and immune cells and these form the basis for the ESTIMATE score to infer tumour purity in tumour tissue. Finally, we describe the biological characteristics of stromal and immune scores in The Cancer Genome Atlas (TCGA) data sets.

**Results**

**Estimation of infiltrating cells and tumour purity.** An overview of ESTIMATE algorithm is shown in Fig. 1. We devised two gene signatures: (1) a 'stromal signature' that was designed to capture the presence of stroma in tumour tissue, and (2) an 'immune signature' that aimed to represent the infiltration of immune cells in tumour tissue (Supplementary Data 1). To generate these signatures, we performed the following steps (Fig. 1). Genes associated with the quantity of infiltrating immune cells in tumour tissue were identified using leukocyte methylation

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**Figure 1 | An overview of the ESTIMATE algorithm.** The ESTIMATE algorithm uses gene expression data to output the estimated levels of infiltrating stromal and immune cells and estimated tumour purity. Infiltrating stromal- and immune cell-related genes were identified by five gene filterings.

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scores, which were previously shown to correlate with the presence of leukocytes in ovarian carcinomas. Gene expression profiles of normal hematopoietic samples were compared with those of other normal cell types. The overlap between the two gene sets constituted the immune signature. Stromal-related genes were selected among non-hematopoiesis genes by comparison of the tumour cell fraction and matched stromal cell fraction after laser-capture microdissection in breast, colorectal and ovarian cancer data sets. Genes with high variability in cancer cell lines and genes highly expressed in glioma stem-like cells were filtered to make up the stromal signature. We used single-sample ssGSEA of these two signatures to generate scores that reflect the presence of each cell type in tumour samples and combined represent a measurement of tumour purity.

In order to evaluate the reliability of the stromal and the immune signatures, we obtained three ovarian carcinoma tumour samples and performed microbead-based cell sorting to separate tumour and non-tumour cell fractions. The epithelial, tumour cell-containing, cell fraction was enriched using an EpCAM antibody. Transcriptional profiles were obtained from the bulk cell-containing, cell fraction after laser-capture microdissection in breast, colorectal and ovarian cancer samples and performed microbead-based cell sorting to separate immune signatures, we obtained three ovarian carcinoma tumour samples and combined represent a measurement of tumour purity.

In the three data sets used in the process of gene selection, there was a significant reduction in the tumour and immune scores in the tumour cell fraction (Fig. 2b; Supplementary Fig. S1). Similarly, the microdissected stroma-enriched fraction in the three independent public data sets, which were not used in construction of the gene signature was significantly decreased (ovarian cancer (GSE29156), breast cancer (GSE10797), lung cancer (GSE33363), P = 2.5 × 10⁻⁵; breast cancer (GSE10797), P = 1.9 × 10⁻⁷; lung cancer (GSE33363), P = 5.7 × 10⁻⁷ by paired t-test; Fig. 2c). Although immune scores in the tumour cell-enriched fraction were lower than those in bulk tumour- or stroma-enriched fraction (ovarian cancer, P = 0.0030; breast cancer P = 3.2 × 10⁻⁷; lung cancer P = 0.0044 by paired t-test; Fig. 2d), one tumour-enriched sample retained a high

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**Figure 2 | Stromal and immune scores for tumour cell and stromal fractions of tumour samples.** Stromal and immune scores were generated using expression data sets obtained from tumour cell or stromal cell-enriched samples. (a,b) Heatmaps display stromal (upper row) and immune score (lower row) per sample (each column) using ovarian cancer samples after (a) microbead-based cell sorting and (b) laser-capture microdissection (red = high, blue = low score). (c,d) Box and whisker plots display reduced (c) stromal and (d) immune scores for the tumour cell-enriched samples (tumour part) after laser-capture microdissection compared with matched stromal cell-enriched (ovary, breast) or bulk tumour samples (lung). Box represents the median (thick line) and the quartiles (line). Whisker expresses 1.5 interquartile range (IQR) of the lower or the upper quartile.
immune score (Fig. 2b), suggesting that immune cells were retained in the microdissected tumour cell-enriched fraction. This observation may reflect the challenges in microdissecting tumour and immune cells that intermix in many tumours. It could also be related to differences between infiltrating immune cells and immune cells surrounding the tumour.4–6

To evaluate the association of the stromal and immune scores with tumour purity, we compared ESTIMATE scores with predictions of tumour purity based on the ABSOLUTE method. ABSOLUTE establishes the fraction of tumour cells in a tumour sample based on somatic DNA copy number alterations and has been shown to provide highly accurate prediction of tumour purity. Immune and stromal signature scores of TCGA Agilent array-based expression profiles of ovarian cancer (n = 417; 28 samples used to define the immune signature were not included in this analysis) showed a significant correlation of both stromal and immune scores with ABSOLUTE tumour purity predictions (Pearson’s correlation coefficient r = 0.65 and −0.60; distance r, 0.65 and 0.58) (Fig. 3a,b). Importantly, ESTIMATE scores showed an increased correlation with tumour purity compared with stromal-only and immune-only scores (Pearson’s r, 0.69; distance r, 0.69) (Fig. 3c). There was a positive correlation between stromal and immune scores (Pearson’s r, 0.62; distance r, 0.58), and samples with low tumour purity showed high stromal and immune scores (Fig. 3d).

Specific samples were associated with high stromal but not high immune scores, and vice versa, suggesting variable infiltrating patterns (Supplementary Data 2).

To illustrate the broad utility of the ESTIMATE algorithm, we applied this model to 10 TCGA tumour types for which both DNA copy number and gene expression data sets were available, profiled on four different platforms (Table 1).24,26–29. These 10 tumour types were among the first cancers to be characterized by TCGA and were included in TCGA’s Pan-Cancer effort. To confirm the accuracy of the ESTIMATE algorithm, receiver operating characteristic (ROC) curve analysis was used to test the accuracy of the ESTIMATE algorithm (Fig. 3e). For example, a cutoff of 0.7 for tumour purity resulted in the ROC curve (AUC) for each cutoff was measured. Equivalent AUC values were explored across different expression platforms as well as different tumour types (Fig. 4a; Supplementary Figs S2–S6).

Immune cells not only infiltrate the tumour cell region but have also been demonstrated to associate with stromal cells, in a cancer-type-specific manner. The correlation between

Figure 3 | The association between tumour purity variables in TCGA’s ovarian cancer data set. (a–d) Scatterplots between tumour purity and (a) stromal, (b) immune, (c) ESTIMATE scores and between (d) stromal and immune scores in the TCGA ovarian cancer data set. TCGA ovarian cancer samples used in the gene selection (n = 28) were not included in the figure. Dash lines denote each median value for stromal and immune scores. (e) The association between tumour purity and stromal- or immune-dominant pattern. Four subgroups were divided based on the median of stromal and immune scores. (f) The ROC curves for four cutoff values in TCGA ovarian cancer data set. N = 417.
stromal and immune scores varied across cancer types, ranging from high (GBM, Pearson’s $r = 0.8$) to modest (KIRC, Pearson’s $r = 0.38$; Fig. 3d; Supplementary Fig. S7). This suggests that the stromal and immune signatures do not measure the same phenotype and reflects the variable association between immune cells and tumour stroma across cancers. Pathology-based estimates of the percentage of tumour cells, stromal cells and infiltrating lymphocytes, evaluated from haematoxylin-eosin-stained slides, were less correlated with ESTIMATE, stromal and immune scores (Fig. 5).

| Table 1 | A list of The Cancer Genome Atlas data sets. |
|---|---|
| Tumour type | Affymetrix | Agilent | RNAseq$^*$ | RNAseqV2$^*$ |
| Bladder urothelial carcinoma | – | – | – | 122 (95) |
| Breast cancer | – | 530 (488) | 774 (723) | 515 (482) |
| Colon and rectal adenocarcinoma | – | 224 (218) | 83 (81) | 264 (255) |
| Glioblastoma multiforme | 529 (417) | 403 (319) | – | 154 (123) |
| Head and neck squamous cell carcinoma | – | 72 (42) | 469 (329) | 480 (329) |
| Clear cell renal cell carcinoma | – | 133 (115) | 155 (130) | 223 (129) |
| Lung adenocarcinoma | – | 230 (228) | – | 262 (248) |
| Lung squamous cell carcinoma | 585 (469) | 558 (442) | – | 333 (253) |
| Uterine corpus endometrial carcinoma | – | 303 (293) | – | 370 (281) |
| Total | 1,247 (1,001) | 1,942 (1,639) | 1,882 (1,515) | 2,920 (2,463) |

The number in parenthesis expresses the number of samples whose tumour purity was calculated by using both gene expression data (ESTIMATE) and copy number data (ABSOLUTE).

*RNAseq and RNAseqV2 are expression data based on Reads Per Kilobase per Million mapped reads (RPKM) and RNA-Seq by Expectation Maximization (RSEM), respectively.

Figure 4 | Evaluation of ESTIMATE algorithm. The accuracy of the ESTIMATE algorithm was evaluated by the AUC when tumour samples were divided into high- and low-purity groups on the basis of DNA copy number-based tumour purity. (a,b) The ROC curves for four cutoff values in (a) the Agilent data set, the Affymetrix data set, and the RNAseq data set, the RNAseqV2 data set, and (b) the validation data set. (c) An example of ESTIMATE for new Affymetrix sample, with an ESTIMATE-predicted tumour purity of 0.58. Black dot and grey dash lines show ESTIMATE tumour purity and 95% prediction interval, respectively. The grey dots represent the background distribution based on 955 samples from the TCGA Affymetrix data set.
In order to facilitate tumour purity prediction using ESTIMATE signatures, we transformed the scoring system to a [0,1] range. First, a regression curve for ESTIMATE score and tumour purity based on ABSOLUTE in the TCGA data set was established. By applying the nonlinear least squares method to the modified TCGA Affymetrix data (n = 995) (Supplementary Fig. S8a), ESTIMATE-based tumour purity prediction model was developed. There was a high correlation between ESTIMATE-based and DNA copy number-based tumour purity (Pearson’s r = 0.74) (Supplementary Fig. S8b).

Validating the capacity of ESTIMATE to predict tumour purity was performed using an independent data set (n = 195) composed of seven publicly available data sets including both Affymetrix microarray expression data and matched SNP array copy number data (Supplementary Table S1). Moreover, ESTIMATE-based tumour purities were highly correlated with the ABSOLUTE-based tumour purities in the independent validation set (Pearson’s r = 0.74) (Supplementary Fig. S8b).

To show the specificity of the tumour purity prediction, we used copy number and expression data from 27 cancer cell lines samples (GSE34211). The root-mean-square error of ESTIMATE and ABSOLUTE were 0.006 and 0.051, respectively, indicating consistent absence of immune and stromal signals (Supplementary Fig. S9). Next, we calculated ESTIMATE scores using the expression profiles from 10 normal ovarian epithelium samples (GSE18520). The ESTIMATE-predicted tumour purity was 0.68 ± 0.12 (Supplementary Table S3), suggesting that normal ovarian epithelium may have some stromal or immune cell components.

We investigated the correlation of the stromal, immune and ESTIMATE scores with methylation-based estimates of the fraction of leukocytes in tumour tissues15. A high correlation between our immune score and leukocyte methylation score was observed across all tumour types (Pearson’s r = 0.75 ± 0.091) (Supplementary Fig. S11). Interestingly, stromal scores were not strongly correlated with leukocyte methylation score (Pearson’s r = 0.51 ± 0.089). These findings showed that our immune scores were specifically associated with the presence of leukocytes across different tumour types.

**Prediction of tumour purity using ESTIMATE.** In order to facilitate tumour purity prediction using ESTIMATE signatures, we transformed the scoring system to a [0,1] range. First, a regression curve for ESTIMATE score and tumour purity based on ABSOLUTE in the TCGA data set was established. By applying the nonlinear least squares method to the modified TCGA Affymetrix data (n = 995) (Supplementary Fig. S8a), ESTIMATE-based tumour purity prediction model was developed. There was a high correlation between ESTIMATE-based and DNA copy number-based tumour purity (Pearson’s r = 0.74) (Supplementary Fig. S8b).

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Patterns of stromal and immune cell scores across different tumour types. Using both TCGA and non-TCGA data sets from 10 different tumour types (Supplementary Table S1), we examined the distribution of stromal and immune score per tumour type (Fig. 6; Supplementary Fig. S12, Supplementary Table S4). As reported previously, lung adenocarcinomas showed lower purity compared with other tumour types\textsuperscript{15}. The relatively high levels of stroma found in clear cell renal cell carcinoma and breast carcinoma may be associated with the high levels of adipocyte content that is characteristic of both tumour types\textsuperscript{35,36}. In high-grade serous ovarian carcinoma, high stromal or immune scores reflect the presence of mesenchymal or immunoreactive gene expression subtypes that have been reported previously\textsuperscript{30,37}. Clear cell renal cell carcinomas are considered to be immunogenic tumours, and this characteristic is captured by the relatively high levels of immune signature expression\textsuperscript{38}. Immuneogenicity is not known as a property of lung squamous cell carcinoma; however, this disease is characterized by a high percentage (\textgreater 95\%) of patients with a history of smoking, which has been linked to lung inflammation\textsuperscript{39,40}. Lung squamous cell carcinomas showed relatively high immune cell scores and have recently been associated with susceptibility to immunomodulatory therapeutics such as ipilimumab\textsuperscript{40}. Further investigation is needed to show that the presence of infiltrating immune cells is a biomarker for immunotherapy response. The similarity in the distribution of stromal and immune scores between lung squamous cell carcinoma and head and neck squamous cell carcinoma suggests that these tumours may harbour a similar genomic profile but also share comparable tumour cellularity\textsuperscript{28}.

The impact of tumour purity on somatic mutations. To examine the impact of tumour purity on the ability to detect genetic alterations, we assigned samples with ESTIMATE scores in the top 25\% to a low-purity subgroup, and samples with the bottom 25\% ESTIMATE scores to a high-purity subgroup, per tumour type. We observed a reduced number of mutations per megabase in low-purity head and neck squamous cell carcinomas and clear cell renal cell carcinomas, (unpaired \textit{t}-test with Benjamini–Hochberg FDR correction, \textit{adjusted} \textit{P} = 0.055 and 0.055) but not in other tumour types, suggesting that the sequencing coverage used for TCGA samples is sufficient to comprehensively detect somatic sequence variants (Supplementary Fig. S13). Next, we evaluated the mutation spectrum of high- and low-purity subgroups by measuring the relative contribution of the two types of transition base substitutions (\textit{A} > \textit{G/G} > \textit{A} and \textit{T} > \textit{C/C} > \textit{T}) and the four classes of transversion base substitutions (\textit{C} > \textit{A/A} > \textit{C}, \textit{C} > \textit{G/G} > \textit{C}, \textit{T} > \textit{A/A} > \textit{T} and \textit{T} > \textit{G/G} > \textit{T}). Two of the ten TCGA data sets (head and neck squamous cell carcinoma, lung squamous cell carcinoma) showed a significantly decreased fraction of \textit{T} > \textit{A} substitutions in the low-purity group compared with the high-purity group (unpaired \textit{t}-test with Benjamini–Hochberg FDR correction, \textit{adjusted} \textit{P} = 0.015 and 0.015, respectively) (Supplementary Table S5). The ratio of transitions and transversions was significantly associated with purity level in head and neck squamous cell carcinoma (adjusted \textit{P} = 0.018).

Discussion

We have developed a new algorithm to infer the level of infiltrating stromal and immune cells in tumour tissues and

Figure 6 | Unique distribution of stromal and immune scores. (a,b) Distinct distributions of (a) stromal and (b) immune scores across different tumour types were observed in RNAseqV2Affymetrix platform data sets. The number of parenthesis means sample size per data sets.
tumour purity using gene expression data. The predictive ability of this method has been validated in large and independent data sets. Genomic, transcriptomic and proteomic analyses using clinical tumour tissue are affected by the fraction of tumour cells present, and methods for evaluation of the non-tumour portions of tumour samples could provide an important context to present, and methods for evaluation of the non-tumour portions of tumour sets. Genomic, transcriptomic and proteomic analyses using this method has been validated in large and independent data sets. Tumour purity of clinical tumour samples is routinely determined by pathologists through visual evaluation of hema-toxylin- and eosin-stained slides. In this study, histological estimates of the percentage of tumour cells, stromal cells and infiltrating lymphocytes did not correlate with well with ESTIMATE, stromal and immune scores, consistent with the weak correlation between DNA copy number-based tumour purity and histological tumour purity. This discrepancy between genomic or transcriptomic-based and pathology-based estimates might be affected by the sensitivity of histopathological examination to interobserver bias and variability in accuracy or the difference in tissue sections in the same sample between nucleic acid extraction and histological evaluation.

The contribution of immune cells to ovarian carcinoma is well recognized, and we chose to use the TCGA ovarian carcinoma samples as the basis for development of the immune signature, as four types of principal information were available: tumour tissue for cell-sorting experiments, estimates of the amount of desmplasia, immunohistochemistry-based counts of the number of leukocytes and methylation leukocyte scores. Importantly, the performance of ESTIMATE in both TCGA and non-TCGA ovarian carcinoma data sets was not distinctively better compared with other tumour types, and we thus believe that the method used to develop the signature is not biased towards ovarian cancers.

The fibroblast/mesenchymal nature of stromal cells separates their gene expression profile from that of the epithelial tumour cells, thus providing a rationale to seek a signature that is characteristic of stromal cells in general, despite the notion that stromal cells may be tumour-type-specific. As expression data sets from three cancer types (ovary, breast and colon) were used to compare tumour cell fractions and matched stromal cell fractions after laser-capture microdissection, we suggest that some of the diversity in tumour-associated stroma among various cancer types was captured. Importantly, the ESTIMATE accuracy among ovarian, breast and colon cancer TCGA samples was not notably better than that of other tumour types, suggesting that the stromal signature can be broadly applied. The dependency of ESTIMATE on infiltrating stromal and immune cells resulted in some limitations, such as the inability to accurately infer tumour cellularity of hematopoietic or stromal tumours (for example, leukaemia, sarcoma and gastrointestinal stromal tumours) because of the high and tumour-intrinsic expression of stromal- or immune-related genes. Owing to the lack of data, we were unable to evaluate ESTIMATE in the context of tumour types such as prostate or pancreas cancer that may present with atypical patterns of tumour-associated cells—that is, increased fractions of normal epithelial cells. Additional methods may be needed to predict cancer cell fractions for such malignancies. The diverse pattern of the presence of stroma and immune cells across tumour types further emphasizes the different context-dependent ways in which tumour-associated normal cells function and more broadly illustrates the impact of the tumour microenvironment on tumorigenesis and homeostasis.

Transitions in tumour cells have been frequently described. It is possible that some overlap exists between the stromal expression signature and a mesenchymal tumour cell phenotype. However, the strong correlation with tumour purity may suggest that epithelial-to-mesenchymal transition is often confused with the increased presence of tumour-associated stroma.

Low tumour purities may reduce the sensitivity of somatic mutation detection. We did not observe an association of tumour purity with mutation rates except in head and neck squamous cell carcinomas and clear cell renal cell carcinoma, suggesting that the impact of tumour purity to identify somatic mutations is less compared with other factors such as depth or coverage or the mutation detection algorithm applied. We noted differences in mutational profile and spectrum between high and low stromal/immune subgroups in several tumour types. The consistent reduction in T > A substitutions in some low-purity cases suggests that the tumour microenvironment can have an impact on mutational processes or alternatively that the types of mutations in the tumour can alter stromal and immune infiltrations. Our ESTIMATE method for the assessment of stromal and immune cells in tumour tissues may provide an additional avenue to increase our understanding of molecular phenotype.

Our results show that the levels of stromal and immune cells in tumour tissue can be associated with clinical characteristics. Further refinement of the lineage characteristics of infiltrating cells, such as distinguishing between various types of leukocytes, may reveal a more consistent pattern of clinical associations than what we have currently described. Novel therapeutics such as ipilimumab and nivolumab alters T-lymphocyte checkpoint control and may be particularly effective in tumours with intrinsically high levels of infiltrating leukocytes. Whether ESTIMATE immune scores could serve as a biomarker for immunotherapy response is a topic for further investigation.

The ESTIMATE method can be applied for assessment of the presence of stromal cells and the infiltration of immune cells in tumour samples using gene expression data. The method is publicly available through the SourceForge software repository (https://sourceforge.net/projects/estimateproject/). The application of ESTIMATE to publicly available microarray expression data sets, as well as new microarray or RNA-seq-based transcriptome profiles, may help in elucidating the facilitating roles of the microenvironment to neoplastic cell and provide new insights into context in which genomic alterations occur.

**Methods**

**Data preparation.** TCGA level 3 gene expression levels were obtained from the TCGA Data Portal in March 2013. In this study, we used 10 tumour types from four platforms: Affymetrix HT-HG-U133A (one-colour type—that is, one RNA sample is labelled with a fluorophore and hybridized to a microarray), Agilent G4502A (two-colour type—that is, one sample and one reference are labelled with different fluorophores and hybridized together on a same microarray), RNAseq (quantified through RNA-seq by Expectation Maximization) (Table 1). The tumour types selected for our study were among the first tumour types analysed through TCGA and were selected as cancer types studied in TCGA’s Pan-Cancer project. In addition, we used 31 data sets of microarray expression or SNP array copy numbers from Gene Expression Omnibus and ArrayExpress, glioblastoma expression data set from the Repository of Molecular Brain Neoplasia Data, cancer cell line expression data set from Cancer Cell Line Encyclopedia (CCLE) and a glioma stem-like cell expression data set from researchers at MD Anderson Cancer Center (Supplementary Table S1).

**Microbead-based cell sorting.** First, the tissue of a fresh frozen ovarian cancer sample was diced into 1-mm pieces. The tissue was further enzymatically dissociated with 0.8 mg/ml HBSS Liberase Research Grade (#05-401-119-001; Roche) and incubated at 37 °C for 1 h, followed by mechanical dissociation using an 18-G needle. To isolate single cells, the resulting cell suspension was filtered using a 40-μm filter. Lastly, the remaining cells were separated into an epithelial tumour cell
fraction and a non-epithelial tumour-associated stromal fraction. For cell sorting, we used antibody-coated microbeads that recognize the epithelial cell surface marker EpCAM (180–0611-101, Miltenyi Biotec) which are detectable by an EpCAM-positive tumour cell fraction and an EpCAM-negative tumour-associated stromal cell fraction. To test the efficiency of our procedure we performed gene expression profiling on three bulk tumours, three EpCAM-positive fractions and three EpCAM-negative fractions after cell sorting using Illumina BeadChip Human HT-12 v4 according to the manufacturer’s instructions for the HT-12 v4 platform. Genes were mapped to a transcript database and combined in one probe set per gene, as described previously. Expression levels from these Affymetrix data sets were individually established using RMA and quantile normalization. Raw data from Affymetrix Human 133 × 3 P array were processed using the Bioconductor rma package with the default gene-set correction. Of the 451 samples from the CCLE expression data set, which was carried out in GeneSpring GX 11.5 (Agilent Technologies) by setting the raw signal threshold to 1.0 and using 75th percentile normalization. Quantile normalization was performed for Illumina Human HT-12 v4 microarray data using the Bioconductor preprocessedCore package. On Affymetrix Human 133 × 3 P array, Agilent G4112F and Illumina Human HT-12 v4 probes measuring the same gene were averaged to obtain one expression value per gene and sample.

**Microarray data processing.** Probes from Affymetrix HG-U133A, HG-U133 Plus2.0 and HT. HG-U133A GeneChip platforms were mapped to a transcript database and combined in one probe set per gene, as described previously. Expression levels from these Affymetrix data sets were individually established using RMA and quantile normalization. Raw data from Affymetrix Human 133 × 3 P array were processed using the Bioconductor rma package with the default gene-set correction. Of the 451 samples from the CCLE expression data set, which was carried out in GeneSpring GX 11.5 (Agilent Technologies) by setting the raw signal threshold to 1.0 and using 75th percentile normalization. Quantile normalization was performed for Illumina Human HT-12 v4 microarray data using the Bioconductor preprocessedCore package. On Affymetrix Human 133 × 3 P array, Agilent G4112F and Illumina Human HT-12 v4 probes measuring the same gene were averaged to obtain one expression value per gene and sample.

**Gene selection.** A flowchart of gene selection in this study is shown in Supplementary Fig. S1. To analyse expression data measured from six different platforms, we extracted 10,412 common genes. In the gene selection process, we used the significance analysis of microarray (SAM) method to detect differentially expressed genes (more than twofold and q < 0.001). Expression levels from these Affymetrix data sets were individually established using RMA and quantile normalization (Supplementary Table S1). Raw data from Affymetrix Human 133 × 3 P array were processed using the Bioconductor rma package with the default gene-set correction. Of the 451 samples from the CCLE expression data set, which was carried out in GeneSpring GX 11.5 (Agilent Technologies) by setting the raw signal threshold to 1.0 and using 75th percentile normalization. Quantile normalization was performed for Illumina Human HT-12 v4 microarray data using the Bioconductor preprocessedCore package. On Affymetrix Human 133 × 3 P array, Agilent G4112F and Illumina Human HT-12 v4 probes measuring the same gene were averaged to obtain one expression value per gene and sample.

ESTIMATE. ESTIMATE outputs stromal, immune and ESTIMATE scores by performing ssGSEA. For a given sample, gene expression values were ranked and rank-ordered. The empirical cumulative distribution functions of the genes in the signature and the remaining genes were calculated. A statistic was calculated by an integration of the difference between the empirical cumulative distribution function, which is similar to the one used in gene set-enrichment analysis but based on absolute expression rather than differential expression.

We developed ssGSEA based on the signatures related to stromal and immune cell infiltration as stromal and immune scores and combined the stromal and immune scores as the ‘ESTIMATE score’. The formula for calculating ESTIMATE-based tumour purity was developed in TCGA Affymetrix data (r = 1,001) including both the ESTIMATE scores and absolute-based tumour purity. To develop a precise prediction model for tumour purity, we excluded six outliers from all Affymetrix data by performing a multivariate outlier criterion based on the generalized extreme studentized deviate test using the Bioconductor Parametric and Resistant Outlier Detection (PARODY) package (Supplementary Fig. S8a). Next, we entered both the ESTIMATE score and tumour purity to Eurosat Formula 0.97 Beta using the default setting. We repeated the analysis but based on absolute expression rather than differential expression.

**HAPSEG and ABSOLUTE.** ABSOLUTE-based tumour purity in the TCGA data sets was obtained from each TCGA working group. To calculate ABSOLUTE-based tumour purity in other data sets, we ran HAPSEG version 1.1.1 and ABSOLUTE version 1.0.4. As indicated on the website, we ran Birdseed v1 using Affymetrix Power Tools and input the resulting apt-probeset-summarize and apt-probeset-sequence files into HAPSEG. After that, we ran ABSOLUTE at the default setting. In the subsequent analyses, we used samples for which the tumour purity levels were called by ABSOLUTE.

**SNP array data for HAPSEG and ABSOLUTE.** We downloaded SNP array data from Gene Expression Omnibus and ArrayExpress. We used Affymetrix CEL files (including per-probe intensity values) from two platforms (Affymetrix GeneChip Human Mapping 250 K Sty array and Genome-Wide Human SNP array 6.0) in this study. Samples that had passed the 93% call-rate threshold (GeneChip Human Mapping 250 K array) or the 86% threshold (Genome-Wide Human SNP array 6.0) were applied to the ABSOLUTE algorithm.

**Leukocyte methylation score.** We downloaded leukocyte methylation signature score data (syn1809223) that predicts the fraction of leucocyte in tumour tissue based on genome-wide DNA methylation data from Synapse BETA and investigated the correlation of stromal, immune and ESTIMATE scores with leukocyte methylation scores for each tumour type.

**Histological purity estimates.** We downloaded Biotab clinical information per sample from the TCGA Data portal. Basically, each tumour specimen was evaluated in optimal cutting thickness sections where tumour heterogeneity was evaluated and a tumour purity score was thereby determined for each tumour type.

**Mutation analysis.** We downloaded mutation annotation format files (syn170680) and mutation rates (syn1713831) based on MiSeq® for 10 different types of tumours from Synapse BETA. From the mutation annotation format files, we extracted mutation status for 10,412 common genes that were used as background in the ESTIMATE algorithm. Of the several mutation types, we used ‘Frame Shift/ Del/Ins’, ‘In_Frame Del/Ins’, ‘Missense_Mutation’ and ‘Nonsense_Mutation’ in this study. We converted the mutation status per gene that was converted into binary data (1, mutated; 0, wild type) to use in the mutation analysis. To examine the impact of infiltrating normal cells on genetic alterations, we extracted high and low ESTIMATE score subgroups from the expression data per tumour type. The high and low ESTIMATE score subgroups were defined, respectively, as those subgroups within the 25th percentile and within the 25th percentile of the ESTIMATE score range. We combined the expression data in the two subgroups with somatic mutation binary data. Samples without either expression or mutation were excluded from this analysis. Mutation frequency was evaluated by the number of mutations per Mbp.

We investigated the impact of multiple subgroups with different mutation frequencies on the number of affected tumour genes and all selected single-nucleotide alterations and converted them into the six classes of base substitution (C>A, C>G, C>T, T>A, T>C and T>G). We then calculated
the relative contribution of each of the six classes of base substitutions and compared them between the two subgroups.

Next, we extracted the respective high and low stromal/immune score subgroups based on the 75th and 25th percentiles of each score per tumour type and combined each subgroup's expression data and mutation data.

Statistical analysis. We conducted all computations with R 2.13.2 (ref. 68) and used standard statistical tests as appropriate. Where appropriate, P-values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate method.

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Author contributions
K.Y. and R.G.W.V. conceived and designed the present study, M.S. performed the experiments. K.Y., H.K. and R.G.W.V. analysed the data. K.Y., R.V., H.K., W.T.-G. and R.G.W.V. developed and coded the ESTIMATE algorithm. E.M., V.T., H.S., P.W.L., D.A.L., S.L.C., G.G., K.S.-H., G.B.M. and TCGA contributed data/materials/analysis tools. K.Y. and R.G.W.V. wrote the manuscript. All authors read and approved the final manuscript.

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