

Identification of prognostic values defined by copy number variation, mRNA and protein expression of LANCL2 and EGFR in IDH1/2-wild-type glioblastoma

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Abstract

Background

Epidermal growth factor receptor (EGFR) and lanthionine synthetase C-like 2 (LanCL2) genes locate in the same amplicon, and co-amplification of *EGFR* and *LANCL2* is frequent in glioblastoma. However, the prognostic value of *LANCL2* and *EGFR* co-amplification, and their mRNA and protein expression in glioblastoma remain unclear yet.

Methods

This study analyzed the prognostic values of the copy number variations (CNVs), mRNA and protein expression of *LANCL2* and *EGFR* in glioblastoma specimens from TCGA database or our tumor banks.

Results

The amplification of *LANCL2* or *EGFR*, and their co-amplification were frequent in glioblastoma of TCGA database and our tumor banks. CNVs of *LANCL2* or *EGFR* were significantly correlated with *IDH1/2* mutation but not *MGMT* promoter methylation status. *LANCL2* or *EGFR* amplification, and their co-amplification were significantly associated with reduced overall survival (OS) of glioblastoma patients, rather than *IDH1/2*-wild-type glioblastoma patients. mRNA and protein overexpression of *LANCL2* and *EGFR* was also frequently found in glioblastoma. *LANCL2*, rather than *EGFR*, was overexpressed in relapsing glioblastoma, compared with newly diagnosed glioblastoma. However, mRNA or protein expression of *EGFR* and *LANCL2* was not significantly correlated with OS of glioblastoma patients. In addition, the intracellular localization of LanCL2, not EGFR, was associated with the grade of gliomas.

Conclusions

Taken together, amplification and mRNA overexpression of *LANCL2* and *EGFR*, and their co-amplification and co-expression were frequent in glioblastoma patients. Our findings suggest that CNVs of *LANCL2* and *EGFR* were the independent diagnostic and prognostic biomarkers for histological glioblastoma patients, but not for *IDH1/2*-wild-type glioblastoma patients.

Background

Glioblastoma multiforme (glioblastoma, GBM), belonging to the highest World Health Organization (WHO) grade glioma (grade IV), is the most common malignant and aggressive primary brain tumor (47.7%) with a high mortality rate (1, 2). According to the 2018 CBTRUS report, the incidence rate of GBM is 3.21 per 100,000 population, which is the highest in malignant brain tumors (2, 3). Under the Stupp's therapeutic protocol (maximal surgical resection followed by adjuvant radiotherapy and chemotherapy with temozolomide), the median overall survival of GBM patients is 14.6 months, while the 2-year overall survival (OS) rate and progression-free survival (PFS) rate is 26.5% and 10.7%, respectively (4). Aberrations of molecular markers such as O(6)-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation, codeletion of 1p and 19q, isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) mutation, telomerase reverse transcriptase (*TERT*) promoter mutation, *TP53* mutation, and epidermal growth factor receptor (EGFR) overexpression show prognostic significance to guide treatment decisions of GBM patients. In particular, *IDH1/2* gene mutations are found in more than 70% of grade II-III glioma and secondary GBM that arises from low-grade glioma. GBM patients with *IDH1/2* gene mutations often have a better clinical outcome than those with wild-type *IDH* (5). *TERT* promoter mutation is found in approximately 80% of patients with primary GBM that develops rapidly without any clinical or histologic evidence of a less malignant precursor lesion. GBM patients with *TERT* mutation often have poor survival and a high risk of death (6).

EGFR, a member of receptor tyrosine kinases (RTKs), is essential to the pathological process in various cancers via activation of PI3K/Akt signaling pathway. Analyzed by The Cancer Genome Atlas (TCGA) database, overall alterations including amplification, mutation, rearrangement and altered splicing of EGFR in GBM are highly frequent (57.4%) (7). Compared with secondary GBM, EGFR amplification (36%) and overexpression (more than 60%) are more common in primary GBM (8, 9). Evidence shows that *EGFR* gene amplification has a strong correlation with EGFR overexpression. Approximate 98% of primary GBM with *EGFR* amplification also exhibit EGFR overexpression, while 70%-90% of those with EGFR overexpression show *EGFR* amplification (8, 10). A number of studies demonstrate that the amplification and overexpression of EGFR are associated with poor prognosis of GBM patients, especially young people (10-12).

However, a retrospective study shows that EGFR amplification is not a prognostic factor for GBM patients treated with surgery. And a meta-analysis also shows that EGFR amplification is not significantly associated with OS of GBM patients, indicating a heterogeneity of significance among difference studies and subjects (13).

Lanthionine synthetase C-like 2 (LanC Like 2, LanCL2), a member of eukaryotic LanC-like protein family, is a homologue of prokaryotic LanC involved in the synthesis of the antibiotic named as lantibiotics (14). LanCL2 is a receptor of abscisic acid (ABA) which is not only a plant hormone but also an endogenous mammalian hormone involved in glycemic control (15). It is also known as testis adriamycin sensitivity protein (TASP) that is able to increase sensitivity of tumor cells to adriamycin via reduction of P-glycoprotein (16). Accumulating evidence show that LanCL2 plays important roles in the regulation of stress response, inflammation and glycometabolism, providing potential target for the treatment of chronic inflammatory, metabolic and immune-related diseases (17, 18). *LANCL2*, along with *SEC61G* and *ECOP* genes, are located in the flank of *EGFR* gene at chromosomal 7p11.2. These genes are in the same amplicon, and their co-amplification with *EGFR* is common in GBM patients (19, 20). However, it is not clear that whether co-amplification of *EGFR* and *LANCL2* has prognostic value for GBM patients, and what are their mRNA and protein expression patterns.

Here, this study analyzed the copy number variations (CNVs), mRNA and protein expression profiles, and their prognostic values of *LANCL2* and *EGFR* in GBM specimens from TCGA database or from the tumor banks of Shenzhen Second People's Hospital and Sun Yat-sen University Cancer Center. We showed that amplification and mRNA overexpression of *LANCL2* and *EGFR*, and their co-amplification and co-expression were frequent in GBM patients. CNVs of *LANCL2* and *EGFR* were the independent diagnostic and prognostic biomarkers for histological GBM patients, but not for *IDH1/2*-wild-type GBM patients. The protein expression pattern and role of LanCL2 were independent to EGFR. LanCL2 overexpression was correlated with glioblastoma recurrence, and its activation may trigger its translocation into the nucleus.

Methods

TCGA database analysis

CNVs and mRNA expression data analyzed using the GISTIC2 algorithm in the TCGA database were achieved in the cBio Cancer Genomics Portal (<http://www.cbioportal.org>) (21, 22). The TCGA Pan-Cancer Atlas dataset involving more than 11,000 human tumors across 33 different cancer types was selected (23-26). The clinical data of GBM and low-grade glioma (LGG) from the TCGA Pan-Cancer database were downloaded to analyze the OS and PFS of the cohort using Kaplan-Meier survival analysis and log-rank test. Chi-square test was carried out to estimate the correlation of CNVs, while the correlation of mRNA expression (RNASeq V2 RSEM) were calculated by Pearson's correlation. CNVs including shallow deletion (possibly heterozygous deletion), diploid, low-level gain and high-level amplification were defined as the putative copy number values of -1, 0, 1 and 2, respectively.

Tumor specimens

Tumor specimens were retrospectively obtained from the tumor banks in the Shenzhen Second People's Hospital and the Sun Yat-sen University Cancer Center. All tumor samples were histologically diagnosed as GBM (WHO grade IV). Four human normal brain tissues (including two craniocerebral trauma, one para-carcinoma and one epilepsy) and four grade I gliomas were used as the negative controls. Identification of all tumor samples or normal brain tissues were confirmed by an experienced pathologist. This study was approved by the Research Ethics Committee of Shenzhen Second People's Hospital and Sun Yat-sen University Cancer Center. All patients were given written informed consent.

DNA extraction and copy number assay

Genomic DNA (gDNA) was extracted using QIAamp DNA Mini Kit (QIAGEN) and copy number variations were evaluated by TaqMan Copy Number Assays (Thermo Scientific) following the manufacturer's instructions. The Taqman Copy Number Assay probes for *LANCL2* (Hs04953915_cn) and *EGFR* (Hs04983302_cn) genes were used for copy number quantitation, while TaqMan Copy Number Reference Assay RNase P was served as the reference. TaqMan Genotyping Master Mix was employed for the PCR amplification procedure, and 20 ng/well of gDNA was added in each PCR reaction, which was performed in ABI Quantstudio™ DX. Each reaction was duplicated.

Western blotting

Total proteins were extracted by RIPA lysis buffer and protein concentrations were determined using the BCA protein assay (Thermo Scientific). Proteins were then separated by 8% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking with 5% non-fat milk or 5% BSA, membranes were incubated with gentle agitation in primary antibodies (1:1000) overnight at 4°C and then in HRP-

conjugated secondary antibodies (1:5000) for 1 hr at room temperature. Positive signals were visualized by ECL chemiluminescence using ChemiDoc MP Imaging System (Bio-Rad).

Immunohistochemistry (IHC)

Tissue microarray slides containing 60 to 80 of paraffin-embedded glioma tissue specimens (Cat.No: HBra-Gli060PG-01 and HBra-G080PG-01) were purchased from Shanghai Outdo Biotech. Slide HBra-Gli060PG-01 included 3 normal brain tissues (1 white matter and 2 cortex), 3 grade I, 9 grade II, 9 grade III, and 34 grade IV gliomas (GBM). Slide HBra-G080PG-01 included 3 normal brain tissues (1 white matter and 2 cortex), 3 grade I, 8 grade II, 22 grade III, and 44 grade IV gliomas (GBM), which shared 54 samples with HBra-Gli060PG-01. Sections were immunostained with appropriate primary antibody and biotin-conjugated goat anti-rabbit IgG. After the detection using DAB detection kit (Booster), slides were counterstained with hematoxylin, dehydrated and mounted. IHC staining scores were calculated as the product of the proportion of positive staining cells (0-4) and the intensity of staining (0-3). The proportion of positive staining cells was graded as followed: 0 (no staining); 1 (1–25%, including 25%); 2 (25–50%, including 50%); 3 (50–75%, including 75%); 4 (>75%). The intensity of staining was graded as followed: negative=0; weakly positive=1; positive=2; strongly positive=3.

Statistical analysis

Data were presented as mean \pm S.E.M and all statistical analyses were carried out using GraphPad Prism 8 software. Relative protein expression was evaluated by measurement of density of Western blotting bands using Image J software. Difference among groups which did not follow a normal distribution was compared using the Mann-Whitney U test or Kruskal–Wallis One-way ANOVA with Dunn's multiple comparisons test. Patients' survival was analyzed using the Kaplan-Meier method and statistical comparisons were evaluated using the log-rank test. The distribution of categorical values within two groups was analyzed by the chi-square test (Fisher's exact test). The difference was considered to be significant at $p < 0.05$. The licenses of software are available under any requirement for permission for use.

Results

Amplification and co-amplification of *LANCL2* and *EGFR* were prevalent in glioblastoma, but were not the independent prognostic markers for *IDH1/2*-wild-type glioblastoma patients.

Firstly, to investigate the CNVs of *LANCL2* and *EGFR* genes in a panel of cancers, 32 studies of different cancer types in TCGA Pan-Cancer Atlas database (n=10967) were selected. Results showed that the dominant genomic alterations of *LANCL2* and *EGFR* in cancers were amplification and mutation, while gene fusion and deep deletion were rare. Glioblastoma, head and neck squamous cell carcinoma, esophagogastric adenocarcinoma and non-small cell lung cancer were the top four tumors with the highest alteration frequencies of *LANCL2* and *EGFR* (Fig.1A-B). Subsequently, two studies Glioblastoma Multiforme (n=592) and Brain Lower Grade Glioma (n=514) were further analyzed. The amplification frequencies of *LANCL2* and *EGFR* in GBM were up to 27.65% (159 of 575 cases) and 44.35% (255 of 575 cases), whereas those in LGG were only 3.91% (20 of 511 cases) and 7.63% (39 of 511 cases), respectively (Fig.1C). The data of LGG contained grade II and III gliomas, including oligodendroglioma, oligoastrocytoma and astrocytoma. Among the LGG data, the amplification frequencies of *LANCL2* and *EGFR* in astrocytoma were the highest (7.33% and 13.92%, respectively), while those in oligoastrocytoma were the lowest (1.07% and 1.60%, respectively) (Fig.1D). Co-amplification of *LANCL2* and *EGFR* was common in GBM, but it was rare in LGG. *LANCL2* amplification was found in 61.96% of GBM samples and 51.28% of LGG samples containing *EGFR* amplification. Furthermore, nearly all GBM and LGG samples containing *LANCL2* amplification displayed *EGFR* amplification (Fig.1E). The main types of *LANCL2* and *EGFR* CNVs in GBM were copy number gain and amplification, whereas shallow deletion and diploid were infrequent. Chi-square test demonstrated a significant correlation between the CNVs of *LANCL2* and *EGFR* ($p < 0.001$) (Fig.1F). We analyzed the top ten genes which had the highest co-amplification frequencies with *LANCL2* or *EGFR*. Results indicated that the amplification frequencies of *EGFR*, *SEC61G* and *VOPP1* genes were the top three highest in *LANCL2*-amplified GBM samples, while *SEC61G*, *LANCL2* and *VSTM2A* were the top three genes co-amplified with *EGFR* (Fig.1G-H). Kaplan-Meier survival analysis was performed to evaluate the prognostic values of *LANCL2* and *EGFR* amplification in GBM patients. Results showed that *LANCL2* and *EGFR* amplification was significantly associated with reduced OS but not PFS of patients with GBM (Fig.1I-L).

The relationship between CNVs of *LANCL2/EGFR* and molecular pathology of GBM samples was analyzed. Wild-type *IDH1/2* was mainly found in GBM samples with *LANCL2/EGFR* gain or amplification. Chi-square test found that CNVs of *LANCL2* or *EGFR* were significantly correlated with *IDH1/2* mutation but not *MGMT* methylation status (Fig.2A-D). To observe whether the prognostic value of *LANCL2/EGFR* amplification was affected by *IDH1/2* mutation status, Kaplan-Meier survival analysis was performed in *IDH1/2*-wild-type GBM patients (n=354). Results found that *LANCL2* or *EGFR* amplification was not significantly correlated with OS and PFS of *IDH1/2*-wild-type GBM

patients (Fig.2E). Similarly, co-amplification of *LANCL2* and *EGFR* was also significantly associated with reduced OS but not PFS of GBM patients. However, in GBM patients with wild-type *IDH1/2*, co-amplification of *LANCL2* and *EGFR* was not correlated with OS and PFS (Fig.2F).

mRNA overexpression of *LANCL2* and *EGFR* were frequent in glioblastoma, but were not associated with the prognosis of glioblastoma patients.

The mRNA expression profiles of *LANCL2* and *EGFR* were investigated in 32 different cancers of TCGA database. In the histograms, the average mRNA expression of *LANCL2* and *EGFR* was organized from lowest to highest priority. Among them, LGG, testicular germ cell carcinoma, GBM and uveal melanoma were the top four tumors with the highest average mRNA expression of *LANCL2*, while the average mRNA expression of *EGFR* was highest in GBM, head and neck cancer, clear cell renal cell carcinoma (ccRCC) and LGG (Fig.3A-B). mRNA overexpression of *LANCL2* and *EGFR* was found in 35.63% (57 of 160 cases) and 48.13% (77 of 160 cases) of GBM samples, respectively (Fig.3.C). However, the mRNA overexpression frequencies of *LANCL2* and *EGFR* in LGG samples were only around 10%, and little difference was shown in astrocytoma, oligoastrocytoma and oligodendroglioma (Fig.3D). The correlation between mRNA expression and CNV of *LANCL2* and *EGFR* was then analyzed. Results showed that mRNA expression of *LANCL2* was significantly elevated in GBM samples with *LANCL2* amplification, compared with GBM samples with diploid or gain of *LANCL2* (Fig.3E). Likewise, the correlation was the same in *EGFR* (Fig.3F). However, mRNA overexpression of *LANCL2* or *EGFR* was not significantly associated with OS and PFS of GBM patients (Fig.3G). Moreover, concurrent mRNA overexpression of *LANCL2* and *EGFR* was found in 26.25% (42 of 160 cases) of total GBM samples and 54.55% (42 of 77 cases) of *EGFR*-overexpressed GBM samples (Fig.3.H). In addition, linear regression analysis demonstrated that mRNA expression of *LANCL2* and *EGFR* was positively correlated ($p < 0.001$) (Fig.3I). Nevertheless, concurrent overexpression of *LANCL2* and *EGFR* was also not significantly correlated with OS and PFS of GBM patients (Fig.3J-K). Interestingly, mRNA expression levels of *EGFR* were significantly elevated in *IDH1/2*-wild-type GBM samples, while no obvious change of *LANCL2* mRNA expression was found, suggesting a significant association between *EGFR* mRNA expression and *IDH1/2* status (Additional file: Figure S1A-B). However, mRNA overexpression of *LANCL2* or *EGFR* was also not significantly associated with OS and PFS of *IDH1/2*-wild-type GBM patients (Additional file: Figure S1C-D).

CNVs, mRNA expression and the prognostic values of *LANCL2* and *EGFR* in low-grade gliomas

To evaluate the prognostic values of *LANCL2* and *EGFR* CNVs and mRNA expression in LGG, the study of LGG (n=514) was analyzed. The dominant type of *LANCL2* and *EGFR* CNVs in LGG was diploid, and chi-square test revealed that the CNVs of *LANCL2* and *EGFR* were significantly correlated ($p < 0.001$) (Fig.4A). The mRNA expression profiles of *LANCL2* and *EGFR* were then investigated, showing that *LANCL2* and *EGFR* mRNA expression was significantly increased when these genes amplified. However, no difference among LGG samples with shallow deletion, diploid and gain of *LANCL2* or *EGFR* was observed (Fig.4B-C). In addition, mRNA expression level of *LANCL2* were also positively correlated with that of *EGFR* in LGG samples (Fig.4D). Similar as the GBM samples, CNVs and mRNA expression of *LANCL2* or *EGFR* were significantly correlated with *IDH1/2* mutation status (Fig.4E-H). All patients with *LANCL2* or *EGFR* amplification contained wild-type *IDH1/2*. These indicated that LGG patients' survival may also be affected by the *IDH1/2* status. Therefore, we evaluated the prognostic values of *LANCL2* and *EGFR* CNVs and mRNA expression in *IDH1/2*-wild-type LGG patients. Unfortunately, results also showed that amplification or mRNA overexpression of *LANCL2* and *EGFR* was not significantly correlated with OS and PFS of *IDH1/2*-wild-type LGG patients (Fig.4I).

Amplification and co-amplification *LANCL2* and *EGFR* were also frequent in glioblastoma from the tumor banks, and were associated with poor overall survival of glioblastoma patients.

To validate the analysis results of TCGA database, we analyzed the copy numbers of 100 GBM patients' samples from our tumor banks by Taqman Copy Number Assay using fluorescent probes targeting *LANCL2* and *EGFR*. The \log_2 copy number values larger than 2 was regarded as amplification. Results showed that compared with the copy numbers in normal brain tissues and grade I gliomas, the copy numbers of *EGFR* were significantly elevated in GBM, while the copy numbers of *LANCL2* had no obvious changes (Fig.5A-B). Interestingly, when the GBM samples were subdivided into newly diagnosed and relapsing tumors, the copy numbers of *LANCL2* and *EGFR* were significantly increased only in newly diagnosed GBM (Fig.5E-F). The amplification frequencies of *LANCL2* and *EGFR* were 62.00% and 55.00% in 100 GBM patients, respectively (Fig.5C, Table.1). *LANCL2* and *EGFR* co-amplification was found in 47.00% of the total GBM samples and 85.45% of GBM samples containing *EGFR* amplification (Fig.5D, Table.1). Pearson's correlation analysis also showed that the copy numbers of *LANCL2* and *EGFR* were positively correlated with each other (Fig.5G). Chi-square tests showed that amplification of *LANCL2* was not correlated with *IDH1* and *TERT* mutations, and *MGMT* methylation, whereas amplification of *EGFR* was significantly associated with *IDH1* and *TERT* mutations. On the other side, co-amplification of *LANCL2* and *EGFR* was not related with

TERT mutation and *MGMT* methylation, but was correlated with *IDH1* mutation (Fig.5H). Without regard to the influence of *IDH1* status, *LANCL2* or *EGFR* amplification, and their co-amplification were significantly associated with decreased OS of GBM patients (n=81) (Fig.5I). However, *LANCL2* or *EGFR* amplification, and their co-amplification were not correlated with OS of *IDH1*-wild-type GBM patients (n=20) (Fig. 5J).

Protein expression and localization of LanCL2 was independent to EGFR in gliomas

To investigate the protein expression profiles of LanCL2 and EGFR, 72 GBM samples (including newly diagnosed and relapsing tumor samples) and 4 low-grade (grade I) glioma samples from our tumor banks were used. Compared with the grade I glioma control, the log₂ relative protein expression values larger than 2 was regarded as overexpression. We found that overexpression of LanCL2 and EGFR was found in 38.89% and 58.33% of the total GBM samples (Fig.6A, Table.2). The protein expression of EGFR was markedly increased in GBM samples, whereas the expression levels of LanCL2 had no significant change (Fig.6B-C). Interestingly, overexpression of LanCL2 was observed in relapsing GBM compared with newly diagnosed GBM (Fig.6D, F). On the other hand, although both the newly diagnosed and relapsing GBM samples displayed elevated EGFR expression compared with the grade I glioma samples, no significant change was found between the newly diagnosed and relapsing GBM samples (Fig.6E-F). Pearson's correlation analysis showed that the expression levels of LanCL2 and EGFR were not correlated (Fig.6G). Chi-square tests showed that overexpression of LanCL2 or EGFR was not significantly associated with *IDH1* or *TERT* mutations, and *MGMT* methylation (Fig.6H). No significant association was also found between the expression of LanCL2/EGFR and OS of GBM patients (Fig.6I-J). Subsequently, we used tissue microarray to investigate the expression pattern of LanCL2 and EGFR in GBM cells. Results also showed that the expression scores of both LanCL2 and EGFR were markedly increased in GBM tissues, compared with normal brain tissues (Fig.7B, D). LanCL2 was expressed in both the normal brain tissues and gliomas. The protein expression level and intracellular localization of LanCL2 were correlated with the grade of gliomas. The higher the glioma grade, the higher the expression intensity of LanCL2. LanCL2 was mainly found in the nucleus and cytoplasm of high-grade glioma cells (grade III-IV), whereas it was expressed on the nuclear membrane of low-grade (grade I-II) glioma cells (Fig.7A). On the other hand, EGFR was barely expressed in the normal brain tissues and low-grade gliomas, but was overexpressed in the grade III-IV gliomas. It was mainly located in the plasma membrane and cytoplasm of both low-grade and high-grade glioma cells (Fig.7C).

Discussion

Amplification and overexpression of *EGFR* are frequently occurred and extensively studied in GBM. Eley et al found that approximate 34% (40 of 118 cases) of GBM samples contained *EGFR* amplification, and 50% (20 of 40 cases) of *EGFR*-amplified GBM samples displayed *LANCL2* co-amplification (19). Concomitant amplification or copy number gain of two genes is a common phenomenon in cancers, such as *MYCN* and *DDX1* in neuroblastoma, *ERBB2/HER2* and *TOP2A* in prostate cancer (27-29). Similar as *LANCL2* and *EGFR*, these genes are located in the same amplification region, which is the driving factor of their co-amplification. Since the co-amplification of *LANCL2* and *EGFR* was found in GBM in 2002, studies of *LANCL2* are barely reported in glioma till now. Only one study using integrative radiogenomic analysis found that the copy number and gene expression of *LANCL2* were significantly increased in multicentric GBM (27). In this study, we firstly analyzed the TCGA database and found that the amplification frequencies of *LANCL2* and *EGFR* in GBM were the highest among 32 different types of tumors, indicating the high specificity of *LANCL2* and *EGFR* amplification in GBM. The frequencies of *LANCL2* and *EGFR* amplification in 575 GBM patients were approximate 28% and 44% respectively, and 62% of GBM samples with *EGFR* amplification contained *LANCL2* co-amplification, which were higher than the frequencies reported by Eley's study. In addition, the amplification frequencies of *LANCL2* and *EGFR* in GBM were six to nine times higher than those in grade II-III gliomas. These findings were verified in 100 GBM samples of our tumor banks, which showed higher amplification and co-amplification frequencies of *LANCL2* and *EGFR* than the results analyzed in TCGA database. Moreover, *EGFR* is the top gene with the highest amplification frequency in the TCGA database of Glioblastoma Multiforme (PanCancer Atlas) (data not shown). These suggest that amplification of *EGFR* or co-amplification of *LANCL2* and *EGFR* were potential diagnostic markers for GBM patients.

Survival analysis of TCGA database and our tumor banks showed that amplification of *LANCL2/EGFR* and their co-amplification were significantly correlated with poor OS, but not PFS of GBM patients. It seems that they were of value for the judgment of the prognosis of histological GBM patients. Subsequently, we found that *IDH1/2* mutation, but not *MGMT* methylation status was correlated with CNVs of *LANCL2* and *EGFR*. However, amplification of *LANCL2/EGFR* and their co-amplification were not associated with the OS and PFS of *IDH1/2*-wild-type GBM patients. Similar studies also find that *EGFR* amplification is closely associated with wild-type *IDH1/2* (28). *CDKN2A/B* deletion, but not *TERT* mutation or *EGFR* amplification, was associated with worse OS and PFS of *IDH*-wild-type GBM patients (29). Our findings indicated that CNVs of *LANCL2* and *EGFR* were not the independent prognostic biomarkers for *IDH1/2*-wild-type GBM patients.

Numerous studies showed that both the mRNA and protein overexpression of EGFR, which were highly correlated with *EGFR* amplification, were the signatures and prognostic predictors for GBM patients (30-33). However, paradox was delineated that the mRNA expression of *EGFR*, not protein expression, showed a close correlation with *EGFR* amplification (34). Currently, no study has yet reported the expression profiles and prognostic values of LanCL2 in GBM. In this study, we found that the mRNA expression levels of *LANCL2* and *EGFR* were positively correlated in GBM of TCGA database. *LANCL2* mRNA expression was significantly increased in *LANCL2*-amplified samples, so was EGFR. In our tumor banks, we found that the protein expression of EGFR was elevated in GBM samples, whereas LanCL2 expression did not significantly change. mRNA and protein overexpression of LanCL2 or EGFR were not associated with poor OS and PFS of GBM patients, let alone in *IDH1/2*-wild-type GBM patients. Although the frequencies of *LANCL2* and *EGFR* amplification and mRNA overexpression in LGG were low, the relationships between CNVs and mRNA expression of *LANCL2* and *EGFR* in LGG were similar with those in GBM. Since the CNVs and mRNA expression of *LANCL2* and *EGFR* were also associated with *IDH1/2* status, the prognostic values of *LANCL2* and *EGFR* in *IDH1/2*-wild-type LGG patients were evaluated. Similar with GBM patients, CNVs and mRNA expression of *LANCL2* and *EGFR* were not the independent prognostic biomarkers for *IDH1/2*-wild-type LGG patients.

Although CNVs, mRNA and protein expression of LanCL2 and EGFR were not the independent prognostic biomarkers for *IDH1/2*-wild-type GBM patients, the roles of LanCL2 and EGFR in GBM cells are of importance and can't be ignored. EGFR is extensively studied in GBM. Plenty of studies showed that EGFR and its active mutant EGFRvIII played critical roles in tumorigenesis, proliferation, angiogenesis, and invasion of GBM (35-38). However, the role of LanCL2 in GBM remains elusive. In this study, LanCL2 protein overexpression was only found in relapsing GBM compared with newly diagnosed GBM, indicating that LanCL2 overexpression may be correlated with GBM recurrence. In contrast, no significant difference of EGFR expression was found between newly diagnosed and relapsing GBM. Moreover, no significant correlation between LanCL2 and EGFR protein expression was showed. These findings suggest the expression pattern and role of LanCL2 in GBM are independent to EGFR. A study demonstrated that the N-terminus of LanCL2 protein could be myristoylated and LanCL2 was located in the plasma membrane, juxta-nuclear vesicles, and the nucleus (39). Here, our immunohistochemical result found that the expression and localization of LanCL2 was correlated with the grade of gliomas. The major localization of LanCL2 in GBM cells was in the nucleus and cytoplasm, whereas it was mainly expressed on the nuclear membrane of LGG cells. Another study showed that LanCL2 is a non-transmembrane G protein-coupled receptor, and its nuclear enrichment was induced by ABA or its unmyristoylation to activate ABA signaling (40). Therefore, we speculated that LanCL2 maintains inactive in the cytoplasm of LGG cells, while LanCL2 transforms to the active form in GBM cells and then translocates into the nucleus.

Conclusion

In summary, this study showed that amplification and mRNA overexpression of *LANCL2* and *EGFR*, and their co-amplification and co-expression frequently occurred in GBM patients, compared with patients with LGG. *LANCL2* or *EGFR* amplification, and their co-amplification were significantly associated with reduced OS of GBM patients, rather than *IDH1/2*-wild-type GBM patients. However, mRNA and protein expression of LanCL2 and EGFR were not correlated with the prognosis of GBM patients. LanCL2 protein was overexpressed in relapsing GBM samples rather than newly diagnosed GBM, whereas overexpression of EGFR was found in both newly diagnosed and relapsing GBM samples. Moreover, the protein expression and intracellular localization of LanCL2 were correlated with the grade of gliomas. LanCL2 was mainly found in the nucleus and cytoplasm of high-grade gliomas (grade III-IV), whereas it was expressed on the nuclear membrane of low-grade (grade I-II) gliomas. Taken together, CNVs of *LANCL2* and *EGFR* were the independent diagnostic and prognostic biomarkers for histological GBM patients, but not for *IDH1/2*-wild-type GBM patients. The protein expression pattern and role of LanCL2 in GBM were independent to EGFR. LanCL2 expression was correlated with GBM recurrence, and its activation may trigger its translocation into the nucleus.

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Shenzhen Second People's Hospital and Sun Yat-sen University Cancer Center. All patients were given written informed consent.

Consent for publication

This study has been approved by all authors for publication.

Availability of data and material

The datasets analyzed during the current study are available in the TCGA repository, <http://www.cbiportal.org>.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HZ analyzed and interpreted all the data, performed the *in-vitro* experiments, was the major contributor in writing the manuscript. XZ performed the follow-up study and collected the clinical information of patients. JW, FC and LC provided the patients' samples and clinical information. CW, PD and LC carried out parts of the *in-vitro* experiments including copy number assay and immunohistochemistry. XW and JL performed the pathologic analysis. ZL, WL, ZC and GH provided suggestions to the study. WL revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations

ABA: abscisic acid; CNV: copy number variation; EGFR: epidermal growth factor receptor; GBM: glioblastoma multiforme; IDH: isocitrate dehydrogenase; IHC: immunohistochemistry; LANCL2: lanthionine synthetase C-like 2; LGG: low-grade glioma; MGMT: O(6)-methylguanine-DNA methyltransferase; OS: overall survival; PFS: progression-free survival; RTK: receptor tyrosine kinase; TASP: testis adriamycin sensitivity protein; TERT: telomerase reverse transcriptase; TCGA: The Cancer Genome Atlas; WHO: World Health Organization;

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Tables

Tables

Table 1. Amplification and co-amplification of *LANCL2* and *EGFR* in GBM samples of tumor banks

Affiliation	NO. of cases <i>LANCL2</i> amplification	NO. of cases <i>EGFR</i> amplification	NO. of cases co- amplification	Total cases	Percent <i>LANCL2</i> amplification	Percent <i>EGFR</i> amplification	Percent co- amplification	Percent co- amplification in <i>EGFR</i> amplification
Shenzhen Second People's Hospital	33	30	27	51	64.71%	58.82%	52.94%	90.00%
Sun Yat- sen University Cancer Center	29	25	20	49	59.18%	51.02%	40.82%	80.00%
Total	62	55	47	100	62.00%	55.00%	47.00%	85.45%

Table 2. Protein overexpression of *LanCL2* and *EGFR* in GBM samples of tumor banks

Affiliation	NO. of cases <i>LanCL2</i> overexpression	NO. of cases <i>EGFR</i> overexpression	Total cases	Percent <i>LanCL2</i> overexpression	Percent <i>EGFR</i> overexpression
Shenzhen Second People's Hospital	19	27	47	40.43%	57.45%
Sun Yat-sen University Cancer Center	9	15	25	36.00%	60.00%
Total	28	42	72	38.89%	58.33%

Figures

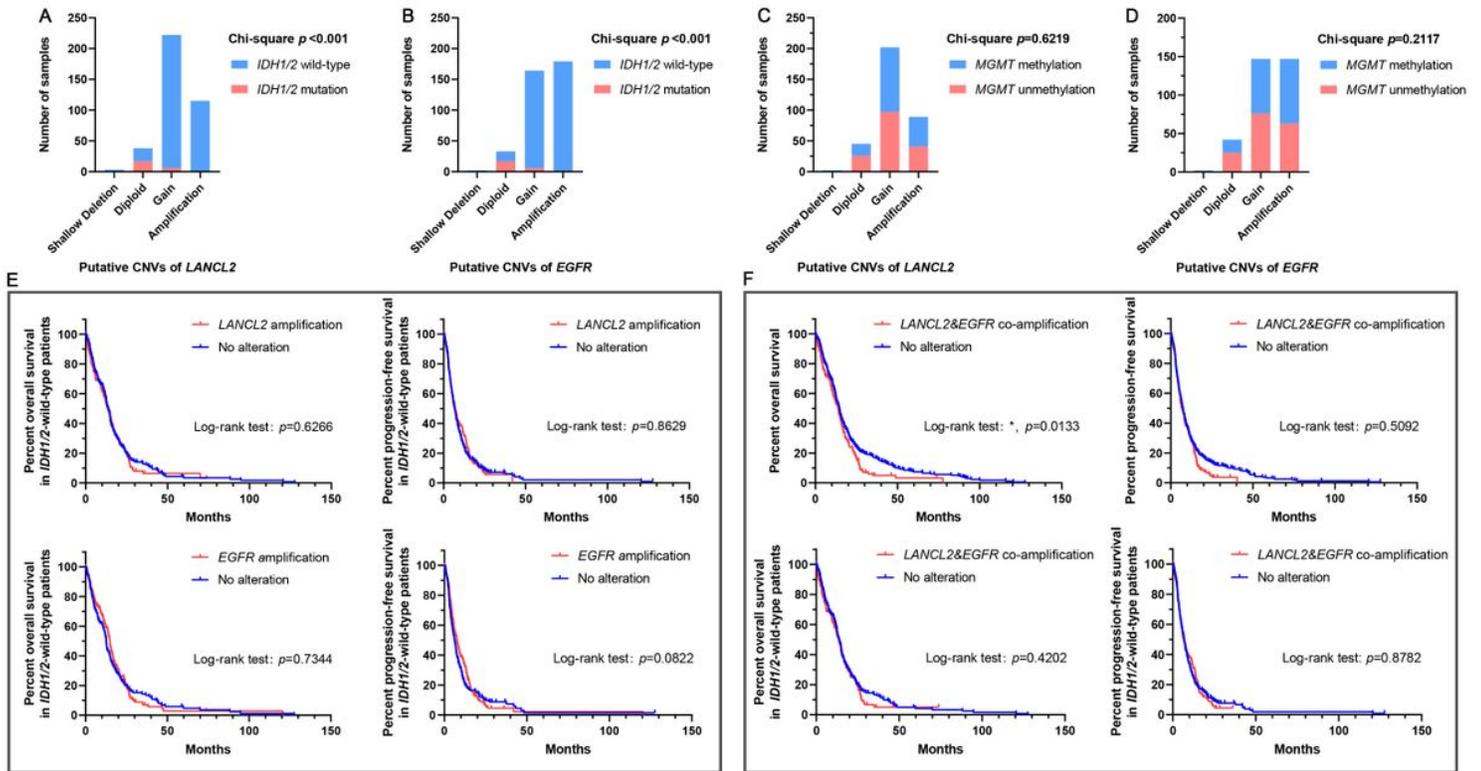


Figure 2

Amplification and co-amplification of *LANCL2* and *EGFR* were not associated with OS and PFS of IDH1/2-wild-type GBM patients of TCGA database. (A-B) Chi-square test showed that CNVs of *LANCL2* and *EGFR* were significantly correlated with IDH1/2 mutation in GBM. (C-D) CNVs of *LANCL2* and *EGFR* in GBM were not correlated with MGMT methylation. (E) Amplification of *LANCL2* and *EGFR* was not correlated with OS and PFS of IDH1/2-wild-type GBM patients. (F) Co-amplification of *LANCL2* and *EGFR* was significantly associated with OS but not PFS of GBM patients, while it was not correlated with OS and PFS of IDH1/2-wild-type GBM patients.

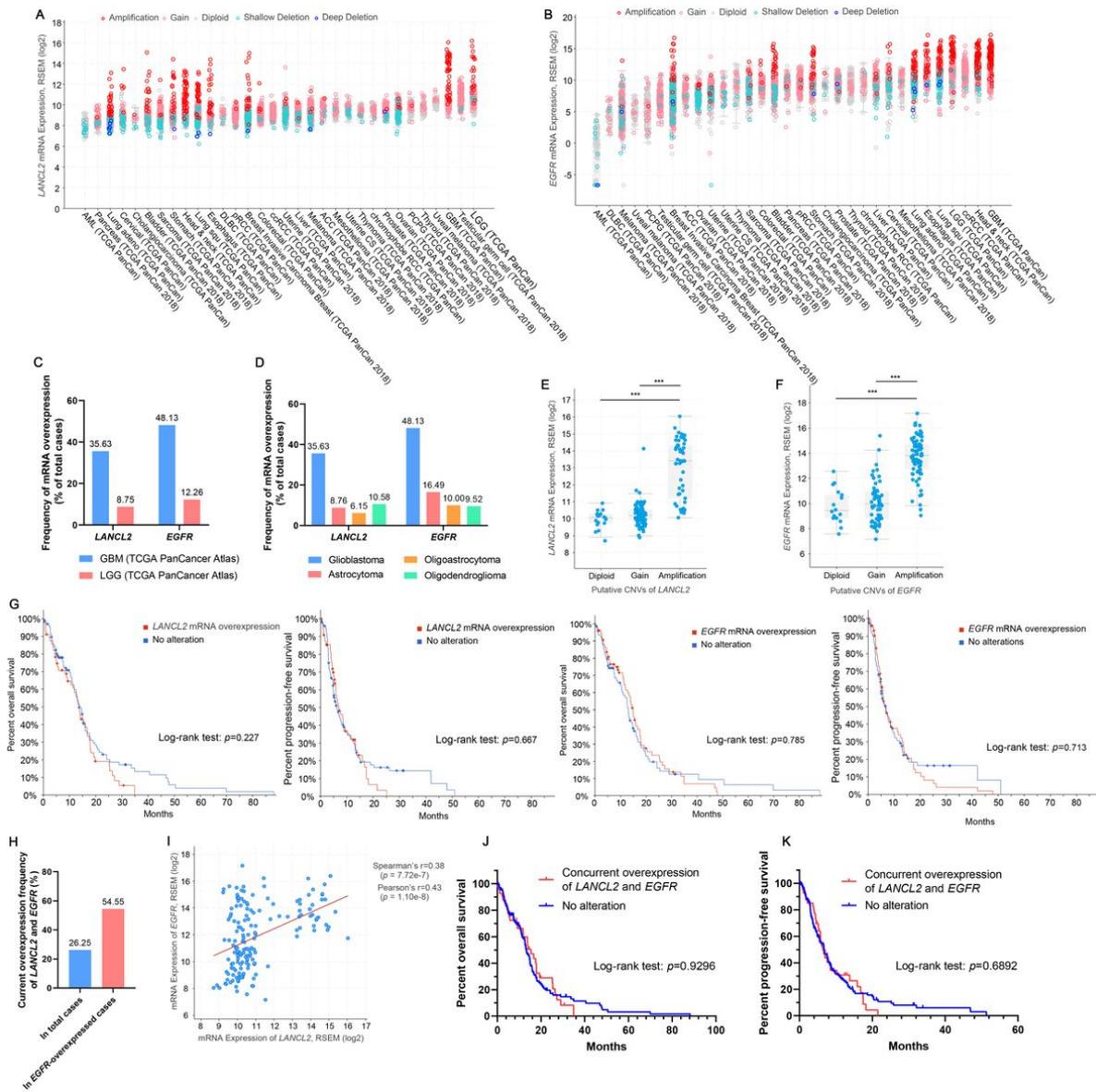


Figure 3

mRNA overexpression of LANCL2 and EGFR was common in GBM specimens of TCGA database, but was not associated with prognosis of GBM patients. (A-B) mRNA expression levels of LANCL2 and EGFR in 32 different cancers of TCGA Pan-Cancer Atlas database. (C) Frequencies of LANCL2 and EGFR mRNA overexpression in two TCGA studies of GBM and LGG. (D) Frequencies of LANCL2 and EGFR mRNA overexpression in different pathological types of gliomas. (E-F) The correlations between CNVs and mRNA expression of LANCL2 or EGFR in GBM. (G) Kaplan-Meier survival analysis showed that mRNA overexpression of LANCL2 or EGFR was not associated with OS and PFS of GBM patients. (H) Frequencies of concurrent mRNA overexpression of LANCL2 and EGFR in total or EGFR-overexpressed GBM samples. (I) A significant association between the mRNA expression levels of LANCL2 and EGFR in GBM. (J-K) Concurrent mRNA overexpression of LANCL2 and EGFR was not correlated with OS and PFS of GBM patients.

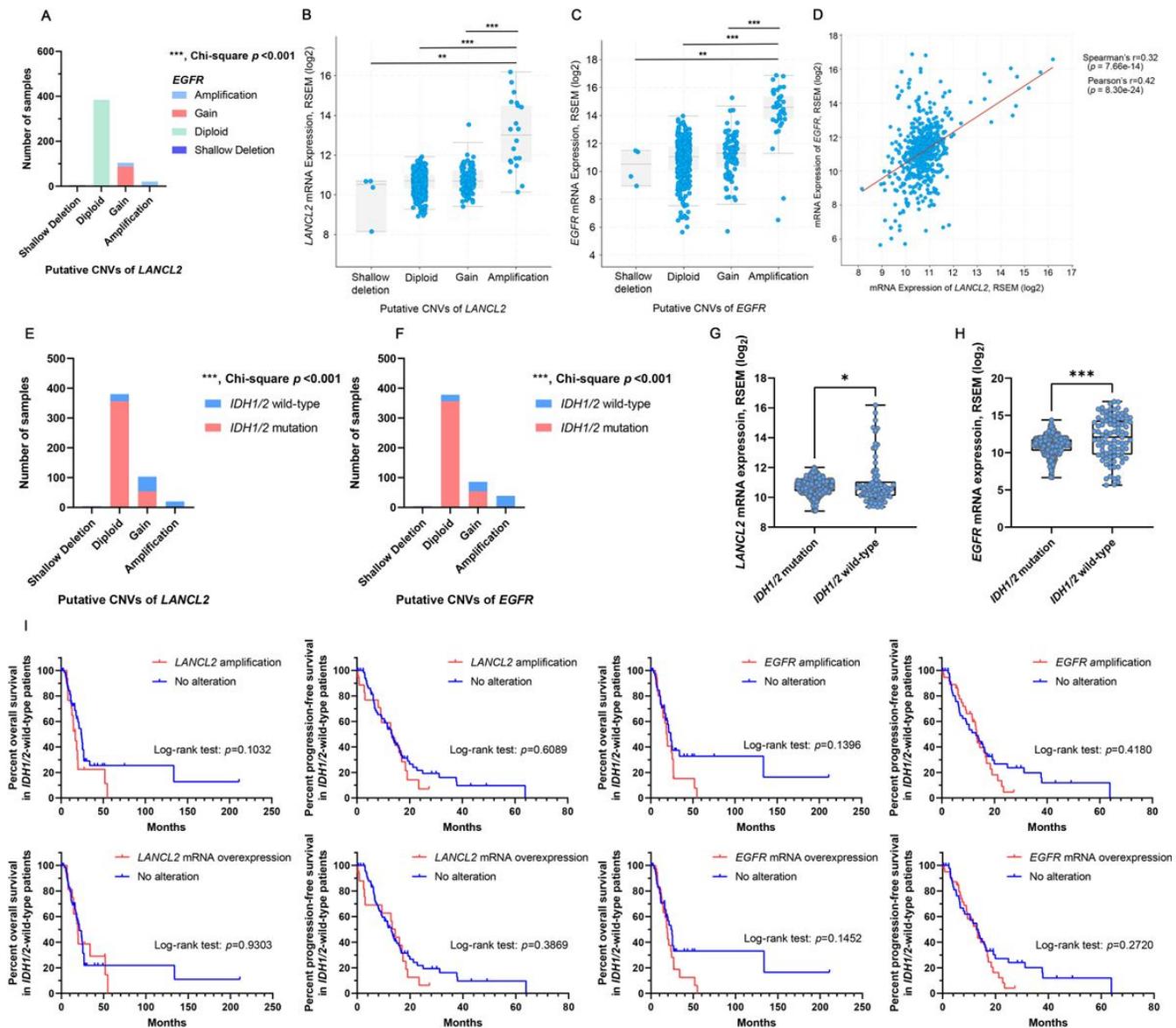


Figure 4

The prognostic values of CNVs and mRNA expression of LANCL2 and EGFR in LGG of TCGA database. (A) Chi-square test showed the CNVs of LANCL2 and EGFR in LGG were significantly associated. (B-C) The correlations between CNVs and mRNA expression of LANCL2 or EGFR in LGG. (D) Correlation analysis showed a significant association between the mRNA expression levels of LANCL2 and EGFR in LGG. (E-F) Chi-square test showed that CNVs of LANCL2 or EGFR in LGG were significantly correlated with IDH1/2 mutation. (G-H) mRNA overexpression of LANCL2 and EGFR in LGG were significantly correlated with IDH1/2 mutation. (I) The prognostic values of amplification and mRNA expression of LANCL2 and EGFR for OS and PFS of IDH1/2-wild-type LGG patients.

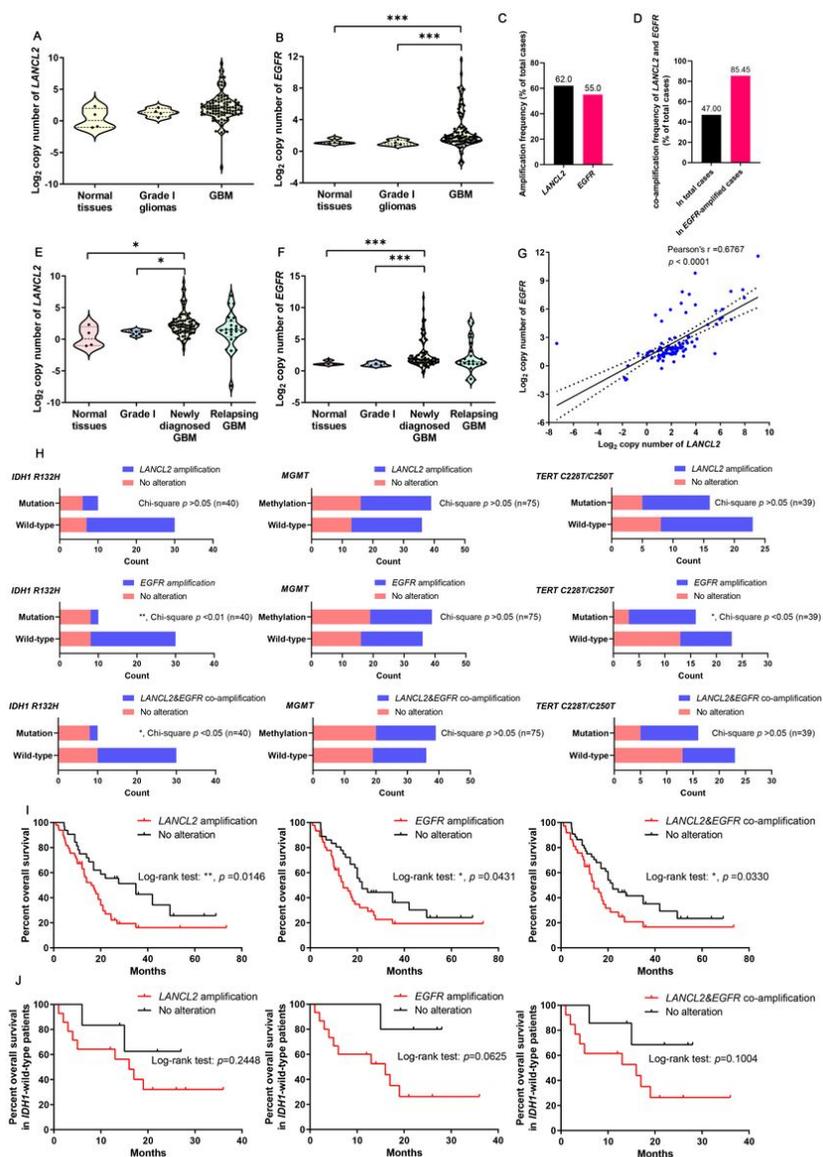


Figure 5

Amplification, co-amplification and their prognostic values of LANCL2 and EGFR were verified in GBM samples of tumor banks. (A-B) Copy numbers of LANCL2 and EGFR in GBM samples of our tumor banks. Normal brain tissues and grade I gliomas were used for comparison. (C) Amplification frequencies of LANCL2 and EGFR in GBM samples of tumor banks. (D) Co-amplification frequencies of LANCL2 and EGFR in total or EGFR-amplified GBM samples. (E-F) Copy numbers of LANCL2 and EGFR in newly diagnosed and relapsing GBM samples. Normal brain tissues and grade I gliomas were used for comparison. (G) Pearson's correlation analysis showed that the copy numbers of LANCL2 and EGFR in GBM were significantly correlated. (H) Chi-square test showed the correlations between the amplification or co-amplification of LANCL2 & EGFR and IDH1/2 mutation, MGMT methylation and TERT promoter mutation. (I) Kaplan-Meier survival analysis showed that amplification and co-amplification of LANCL2 and EGFR were significantly correlated with OS of GBM patients (*, $p < 0.05$; **, $p < 0.01$). (J) The prognostic values of amplification and co-amplification of LANCL2 and EGFR in IDH1/2-wild-type GBM patients.

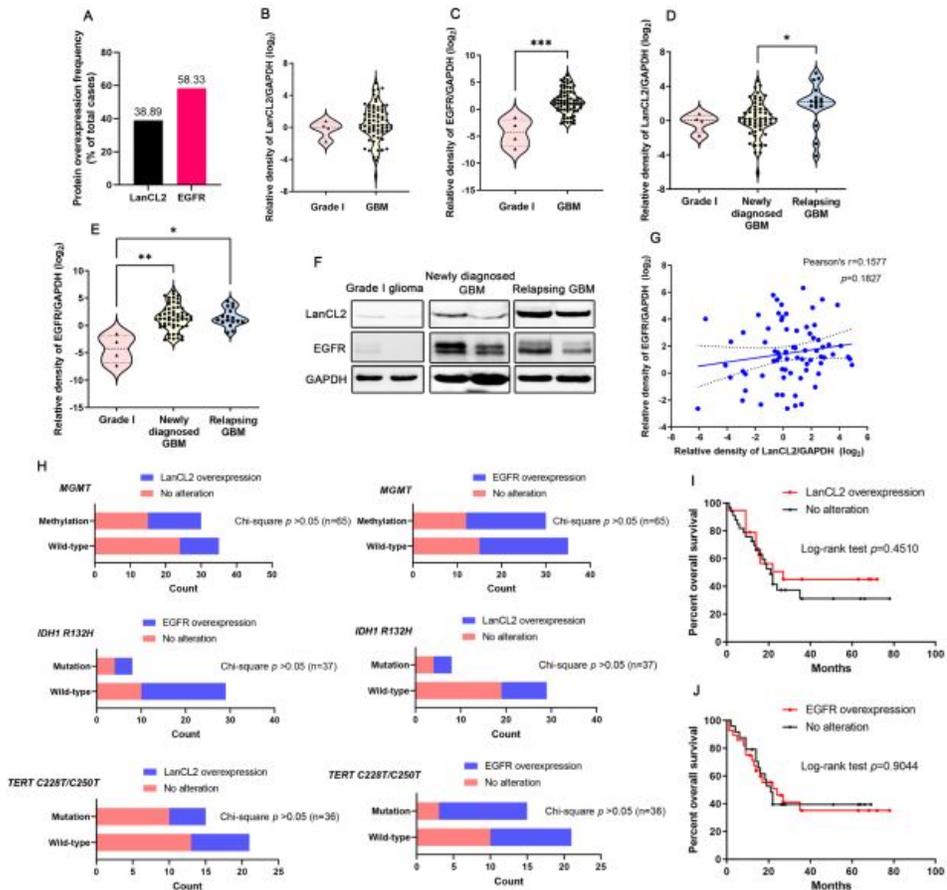


Figure 6

Protein expression profiles and prognostic values of LanCL2 and EGFR in GBM samples of tumor banks. (A) Frequencies of LanCL2 and EGFR protein overexpression in GBM samples of our tumor banks. (B-C) Relative expression of LanCL2 and EGFR in GBM samples was evaluated by measurement of density of immunoblotting bands. (D-E) Relative expression of LanCL2 and EGFR in newly diagnosed and relapsing GBM samples. Grade I gliomas were used for comparison, and GAPDH was served as the endogenous control. (F) Representative immunoblots of LanCL2 and EGFR protein expression in newly diagnosed and relapsing GBM specimens. (G) Person's correlation analysis showed that protein expression of LanCL2 and EGFR was not significantly correlated. (H) Chi-square test showed the correlations between the protein expression of LanCL2 & EGFR and IDH1/2 mutation, MGMT methylation and TERT promoter mutation. (I-J) Protein overexpression of LanCL2 and EGFR was not significantly correlated with OS of GBM patients.

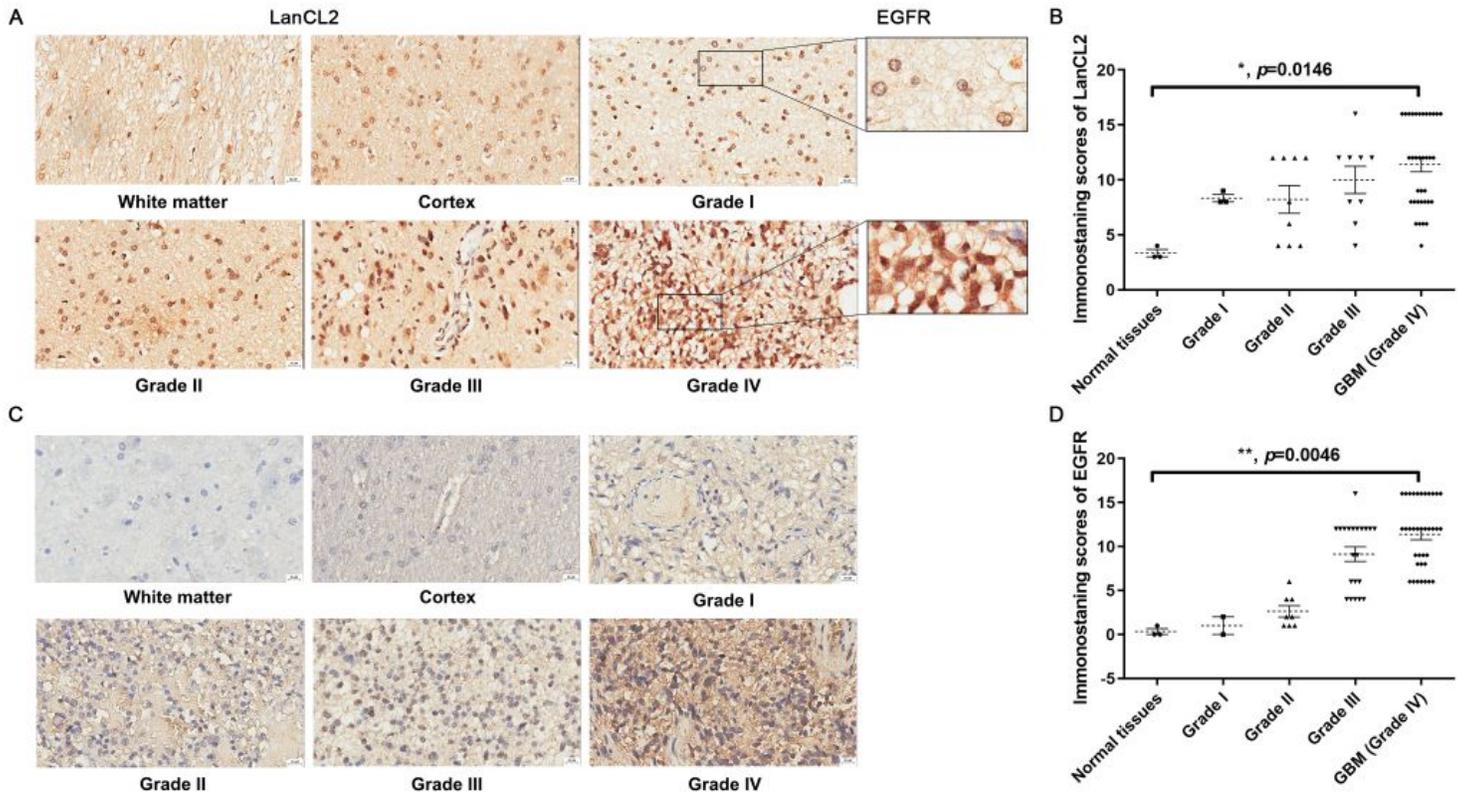


Figure 7

Protein expression and intracellular localization of LanCL2 was correlated with the grade of gliomas. (A) Immunohistochemistry analysis of LanCL2 in representative sections of grade I to IV gliomas. Sections of white matter and cortex were used for comparison. Bar = 20 μ m. (B) IHC staining scores of LanCL2 in tumor sections. (C) Immunohistochemistry analysis of EGFR in representative sections of grade I to IV gliomas. Bar = 20 μ m. (D) IHC staining scores of EGFR in tumor sections. P values were determined by One-way ANOVA and Post Hoc multiple comparison Tukey HSD test. *: $p < 0.05$; **: $p < 0.01$.

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