Mutations in the helix αC of the catalytic domain from the EGFR affect its activity in cervical cancer cell lines

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Abstract. The EGFR is a protein that belongs to the ErbB family of tyrosine kinase receptors. The EGFR is often over-expressed in human carcinomas. Amplification of the EGFR gene and mutations in the EGFR tyrosine kinase domain occur in patients with cancer. In cervical cancer, the expression level of the EGFR protein appears to directly associate with human papillomavirus infection. Our previous research demonstrated that in the cervical cancer cell lines, CALO and INBL, the EGFR is non-phosphorylated. The aim of the current study was to analyze the catalytic activity of the isolated EGFR and the presence of mutations in the control region αC. Catalytic activity was assessed by a universal *in vitro* kinase assay using polyGluTyr as a substrate, and the proteins were visualized by western blotting. For mutation analysis, DNA from CALO and INBL cell lines was isolated, and PCR was used to amplify the exons corresponding to the helix αC in the EGFR. The PCR products were visualized by agarose gel electrophoresis. The bands were isolated using a Zymoclean Gel DNA Recovery kit and directly sequenced. The EGFR, which was isolated and analyzed using the *in vitro* kinase assay, had catalytic activity. The receptor contained some mutations in the helix αC of the catalytic domain in both cell lines. The observed changes in the amino acid sequence may induce a different spatial arrangement and, therefore, a different conformation, which may confer different activities to this receptor. Thus, it was concluded that non-phosphorylated EGFR has catalytic activity, and it bears some amino acid changes in the helix αC of the catalytic domain in the CALO and INBL cells. These results suggest that the EGFR may function as an activator of other ErbB family receptors in these cervical cancer cells.

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Introduction

Cervical carcinoma is one of the most common malignancies in women from Mexico, representing the second most common female neoplasm, in 2020 the incidence rate was 6.5% and the mortality rate was 7.7% (1). Epidemiological and molecular data indicate that persistent infection with high-risk human papillomavirus (HPV) is a risk factor for cervical cancer development and has been associated with other pathologies, such as head and neck, and anal cancers (2,3). HPV DNA is present in virtually all cervical cancer and precursor lesions (4). The expression of the EGFR protein has been associated with HPV infection, as evidenced by its increasing expression level as the grade of intraepithelial neoplasia increases (5). The HPV16 E5 oncogene cannot transform keratinocytes by itself, but it increases the efficiency of cell immortalization by E6/E7 (6). The E5 protein inhibits the downregulation of the EGFR receptor in the presence of the ligand, increasing the steady-state level of EGFR on the cell surface (7).

The EGFR is part of the ErbB family of tyrosine kinases receptors; the other members are HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. The activation of the EGFR leads to autophosphorylation and this change initiates a cascade of downstream signaling pathways. The activity of the EGFR initiates an allosteric interaction between the kinase domain, in an asymmetric dimer, and the helix αC, which is an important element in kinase regulation. The conformation of the helix αC defines an inactive conformation that is characteristic of numerous kinases. In the active conformation, the helix αC has a conserved glutamate, which is able to form a salt bridge with a lysine that coordinates ATP (8-11).

A significant change in the EGFR results from the presence of mutations in the intracellular tyrosine kinase domain. These mutations are restricted to the first four (exons 18-21) of the seven exons that encode the tyrosine kinase domain.

Previous studies have demonstrated that these small mutations prolong the activity of ligand-activated receptors (12-17). In addition, previous studies have also identified somatic mutations or evidence of gene amplification (13,17); however, activating mutations in exons 19-21, from the EGFR, have not been identified in cervical cancer (18).

Our previous research demonstrated that the EGFR was not phosphorylated in the CALO and INBL cervical cancer cell lines (19). Since the receptor was not phosphorylated we hypothesized that it was a dead kinase due to the presence of inactivating mutations. Therefore, the aim of this study was to analyze the catalytic activity of the isolated EGFR as well as the presence of mutations in the control region αC of the catalytic domain.

Materials and methods

Cell culture conditions. The HPV-associated cervical cancer cell lines, CALO, INBL (cell lines established in the Cell Differentiation and Cancer Research Unit, FES Zaragoza, National University of Mexico, Mexico City, Mexico) (20), HeLa [cat. no. American Type Culture Collection (ATCC)-CCL-2] and CasKi (cat. no. CRM-CRL-1550), as well as the THP1 cell line (cat. no. ATCC TIB-202) (all purchased from ATCC), were cultured in RPMI-1640 (Microlab Industrial) medium supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). All the cell lines were incubated at 37˚C in a humidified incubator with 5% CO_2. The cells were treated with 20 ng/ml EGF for 5 min.

Cell lysis. The cell lines were lysed with ice-cold lysis buffer [1% Triton X-100, 5 mM EDTA, 140 mM NaCl, 50 mM Tris (pH 7.4), 1 mM phenylmethylsulfonylfluroide, 1 mM NaF, 1% aprotinin, 1 μM leupeptin, 1 μM pepstatin and 100 μM Na_3VO_4] for 15 min. The lysates were then centrifuged at 1,363 x g at 4˚C for 15 min and the supernatants were then collected.

Immunoprecipitation and immunoblotting. For immunoprecipitation, treated or untreated cells were lysed as aforementioned. The total protein content of the lysates was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc.) and 150 μg protein was incubated with protein A-agarose beads (Invitrogen; Thermo Fisher Scientific, Inc.) previously coupled with 0.3 μg anti-EGFR (cat. no. sc-53274) or anti-HER2 (cat. no. sc-284) antibodies (Santa Cruz Biotechnology, Inc.) for 3 h at 4˚C. The immunoprecipitated proteins were washed five times with ice-cold lysis buffer, resolved with 10% SDS-PAGE and transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad Laboratories, Inc.). The membranes were blocked in TBS with 0.1% Tween-20 and 3% bovine serum albumin (Santa Cruz Biotechnology, Inc.) overnight at 4˚C.

The membranes were analyzed using both mouse anti-phosphotyrosine antibodies [pY20 (cat. no. sc-508) and pY99 (cat. no. sc-7020); Santa Cruz Biotechnology, Inc.] for 2 h, followed by incubation for 45 min with HRP-conjugated rabbit anti-mouse antibody (cat. no. 31450; Thermo Fisher Scientific, Inc.) at room temperature (dilutions, 1:10,000). The proteins were visualized using an enhanced chemiluminescence detection system (Super Signal; Pierce; Thermo Fisher Scientific, Inc.). To determine the presence of EGFR and HER2 in the same membrane, the anti-phosphotyrosine antibodies were stripped. After stripping the membrane with 0.1 M glycine (pH 2.5) and blocking with 5% bovine serum albumin overnight, the same membranes were incubated with the anti-EGFR (1:1,000) or anti-HER2 (1:1,000) antibodies (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature, then treated as mentioned above. All figures show representative results from at least three independent experiments.

In vitro kinase assay. For the *in vitro* kinase assay, a Universal kinase assay kit (fluorometric) (Abcam) was used, according to the manufacturer's instructions. Briefly, $3x10^6$ from each cell line, treated or untreated, were lysed with ice-cold lysis buffer [1% Triton X-100, 5 mM EDTA, 140 mM NaCl, 50 mM Tris (pH 7.4), 1% aprotinin, 1 μM leupeptin, 1 μM pepstatin] for 15 min. The lysates were centrifuged at 1,363 x g at 4˚C for 15 min, then the supernatants were collected and the EGFR receptor was immunoprecipitated as aforementioned. The immunoprecipitated proteins were washed three times with ice-cold lysis buffer, three times with ice-cold PBS (pH 7.4), then three times with 50 mM Tris (pH 7.4).

To each tube, 20 μl ADP assay buffer, 20 μl ADP sensor buffer, 10 μl ADP sensor, 1 μM ATP and 0.03 μg poly-GluTyr poly amino acid (Sigma-Aldrich; Merck KGaA), as the substrate, were added to a total ADP assay volume of 50 μl/sample. For the *in vitro* kinase assay without substrate, the polyGluTyr was omitted. The reaction mixture was incubated at room temperature for 30 min. The fluorescence intensity was measured at excitation/emission, 540/590 nm in black plates in a Fluoroskan Ascent Fluorometer (Thermo Fisher Scientific, Inc.).

Finally, the HeLa cell line was used as a control for catalytic activity of EGFR and the myelomonocytic THP1 cell line was used as a negative control.

DNA extraction and sequencing. For DNA extraction, DNAzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used, according to the manufacturer's specifications. Briefly, $3x10^6$ from each cell line was lysed with 0.75 ml DNAzol, then the lysates were centrifuged (10,000 x g at 4˚C for 10 min) and the supernatant was transferred to a new tube. Subsequently, the DNA was precipitated following the addition of 0.5 ml 100% ethanol. The samples were centrifuged at 4,000 x g at 4˚C for 2 min, then the supernatant was discarded. The obtained DNA was washed twice with 70% ethanol, air-dried for 20 sec, then dissolved in DNase free water.

The obtained DNA was amplified using PCR. DreamTaq DNA polymerase (cat. no. EP0702; Thermo Fisher Scientific, Inc.) was used and the following primers were used: αC helix forward, 5'-CGTAAACGTCCCTGTGCTAGG-3' and reverse, 5'-CCTTATCTCCCCTCCCCGTAT-3'; and GAPDH forward, 5'-GGGACGCTTTCTTTCCTTTCGC-3' and reverse, 5'-GTCGGGTCAACGCTAGGCTG-3' (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used as the internal control. The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; 58˚C for 1 min; 72˚C for 1 min and 95˚C for 1 min for 30 cycles. The product was visualized using agarose gel (1.5%) electrophoresis and the DigiDoc-It transilluminator (Analytik Jena GmbH). The

bands corresponding to the expected products were excised and purified with a Zymoclean Gel DNA Recovery kit (Zymo Research Corp.) and sent for sequencing using the Sanger method to the Faculty of Higher Studies (Facultad de Estudios Superiores) Iztacala facilities at National University of Mexico (Mexico City, Mexico) (ABI Prism 7500; Applied Biosystems; Thermo Fisher Scientific, Inc.).

Modelling of amino acid sequence. The putative amino acid sequences for EGFR in CALO and INBL cells, obtained from Basic Local Alignment Search Tool (BLASTx) for proteins (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), were modeled using SWISS-MODEL Homology Modeling (https://swiss-model.expasy.org/interactive). SWISS-MODEL is a fully automated protein structure homology-modelling server. The SWISS-MODEL Workspace is a web-based working environment, where several modelling projects can be carried out. Protein sequence and structure databases necessary for modelling are accessible from the workspace and are updated in regular intervals. Tools for template selection, model building and structure quality evaluation can be invoked from within the workspace directly or via the web page menu. Building a homology model comprises four steps: i) Identification of structural template(s); ii) alignment of target sequence and template structure(s); iii) model-building; and iv) model quality evaluation (21-23).

Statistical analysis. All the data were obtained from three independent experiments for statistical analysis. The data are presented as the mean ± standard error of the mean. An unpaired Student's t-test (two-tailed) for parametric data was used to compare treatment groups using the GraphPad Prism v8.0.1 statistical package (GraphPad Software, Inc.). P-values and 95% confidence intervals were calculated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Presence of the EGFR in the cervical cancer cell lines. Our previous research demonstrated that the cervical cancer cell lines, CALO and INBL expressed the EGFR; however, this molecule was not phosphorylated (19). The CALO, INBL, HeLa and CasKi cell lines were lysed to determine the presence of the EGFR in the cervical cancer cells, and the proteins were immunoprecipitated with protein-A agarose beads coupled with an anti-EGFR antibody, resolved using SDS-PAGE and transferred to nitrocellulose membranes. The results showed that the EGFR was present in all the cell lines; however, it was not phosphorylated in the CALO and INBL cell lines (Fig. 1).

EGFR is present in the CALO and INBL cervical cancer cell lines, but does not co-precipitate with HER2. As HER2 was also found to be present in the CALO and INBL cell lines (19), the ability of the EGFR to interact with HER2 was analyzed using a co-precipitation assay. The results showed that HER2 does not co-precipitate with the EGFR or vice-versa (Fig. 2).

Non-phosphorylated EGFR is present in the CALO and INBL cell lines and is catalytically active. Our previous research

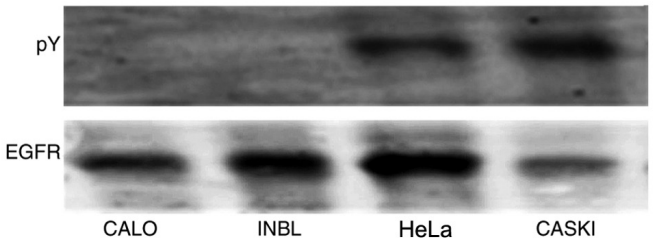

Figure 1. EGFR is present in different cervical cancer cell lines. A total of $3x10^6$ cells were lysed and the receptor was immunoprecipitated using an anti-EGFR antibody. Western blot analysis was then performed using anti-pY antibodies and the membranes were stripped and re-probed for EGFR. pY, phosphotyrosine.

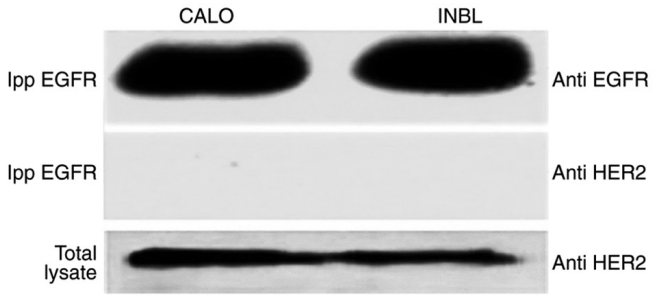

Figure 2. EGFR does not co-precipitate with HER2 in the CALO and INBL cervical cancer cell lines. A total of $6x10^6$ cells were lysed and EGFR was immunoprecipitated using an anti-EGFR antibody. Western blot analysis was then performed using an anti-HER2 antibody, and the membranes were stripped and re-probed for EGFR. Ipp, immunoprecipitated.

demonstrated that the EGFR was not phosphorylated in the CALO and INBL cell lines (19), thus to determine the catalytic activity of the EGFR, the CALO, INBL, HeLa, CasKi and THP1 cell lines were stimulated with EGF. After 5 min, the cells were lysed, and the proteins were immunoprecipitated with protein A-agarose beads coupled with an anti-EGFR antibody, then the catalytic activity was determined using a Universal kinase assay kit.

Surprisingly, it was found that the EGFR receptor isolated from the CALO and INBL cell lines showed high catalytic activity (Fig. 3A). The EGFR, present in the CALO cell line, had similar catalytic activity to the receptor in the HeLa cell line; however, the INBL cell line had a reduced EGFR catalytic activity. The EGFR present in the CasKi cell line had the same catalytic activity with or without EGF treatment. The myelomonocytic THP1 cell line was used as a negative control; however, the receptor was present in this cell line, and a slight increase in the activity of EGFR was observed when treated with EGF (Fig. 3A).

To analyze the autophosphorylation activity of the receptor an *in vitro* kinase assay without substrate was designed (Fig. 3B). The catalytic activity of the EGFR in all the cell lines was lower than the activity observed in the presence of the substrate. The EGFR showed similar catalytic activity in the CALO, HeLa and CasKi cell lines; however, the basal activity of the EGFR in the CALO cell line was higher compared with the HeLa cell line. When the cells were stimulated with EGF, the catalytic activity of the receptor in the CALO and HeLa cell lines increased at the same level; however, in the

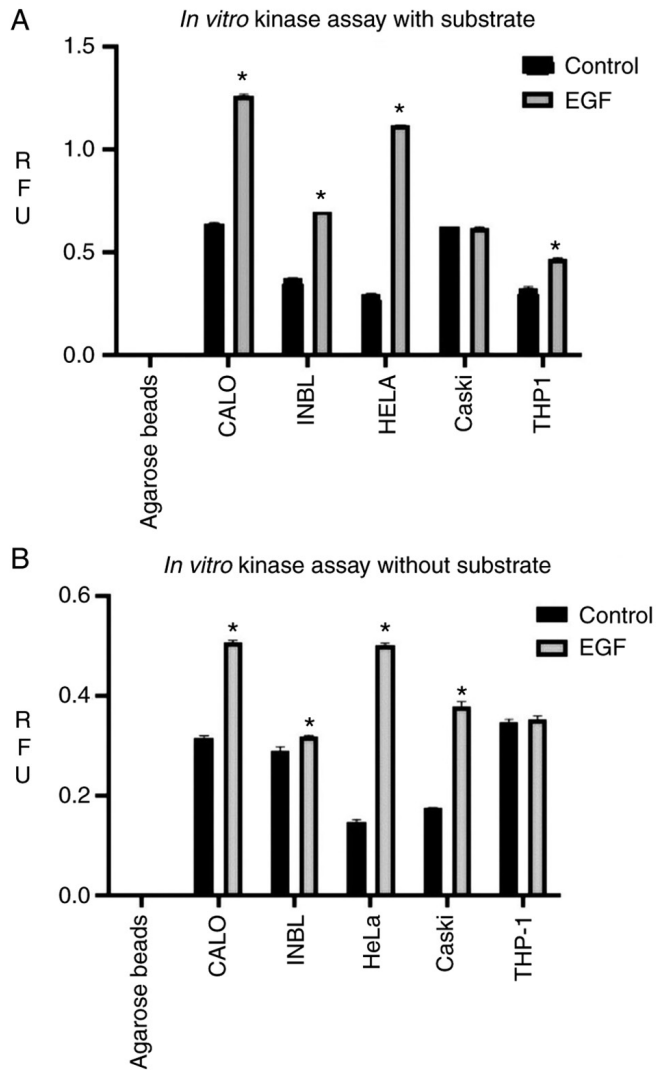

Figure 3. EGFR is present in the CALO and INBL cell lines and has catalytic active. A total of $3x10^6$ cells, treated or untreated with EGF, were lysed and the receptor was immunoprecipitated. The isolated EGFR was analyzed using an *in vitro* kinase assay (fluorometric) (A) with or (B) without substrate. The results are presented as the mean ± SEM from three independent experiments. $^*P<0.05$.

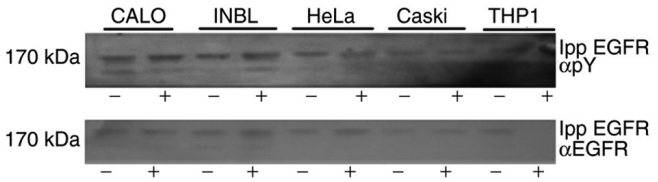

Figure 4. Tyrosine phosphorylation analysis of the EGFR present in the cervical cancer cell lines. The EGFR coupled to agarose beads, used to determine the kinase activity in the absence of the substrate, was separated using 10% SDS-PAGE, then incubated with an anti-phosphotyrosine antibody to determine the presence of pEGFR (upper). The membrane was stripped and re-probed for EGFR (bottom). -, Non-treated cells; +, treated cells with EGF; ipp, immunoprecipitated; pY, phosphotyrosine.

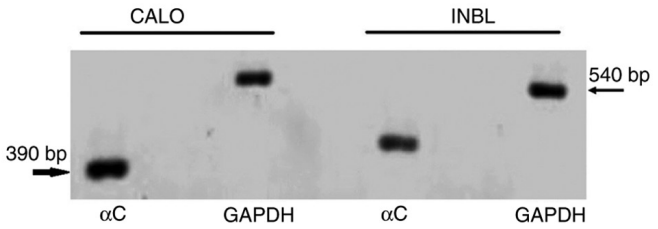

Figure 5. Determination of the αC domain from the EGFR in the CALO and INBL cell lines. The DNA was extracted using DNAzol, then amplified using PCR. The product was visualized using 1.5% agarose gel electrophoresis. Arrows indicate the size of the PCR product.

CasKi cell line, the receptor showed an increase in catalytic activity (75%) compared with that in the HeLa cell line (Fig. 3B). In the assay without substrate, only THP1 cell line did not have an increase in the catalytic activity of the EGFR protein with or without EGF treatment.

The immunoprecipitated EGFR, used to determine the enzymatic activity without a substrate, was separated using SDS-PAGE to analyze its autophosphorylation ability (Fig. 4). The results showed the presence of the phosphorylated receptor in all the cell lines (Fig. 4, upper panel), indicating that the isolated EGFR had catalytic activity and it could phosphorylate its tyrosine residues. After the membrane was stripped, it was re-probed with anti-EGFR antibodies, which confirmed the presence of EGFR (Fig. 4, bottom panel).

Sequence determination of the αC domain in the EGFR isolated from the CALO and INBL cell lines. Our previous research demonstrated that the EGFR was not phosphorylated in the CALO and INBL cell lines (19), and a possible explanation for the lack of tyrosine phosphorylation of the EGFR in the CALO and INBL cell lines is the presence of mutations. Therefore, possible mutations in the catalytic domain of the receptor were investigated. DNA from the CALO and INBL cell lines was isolated and PCR was used to amplify the exons corresponding to the αC helix in the EGFR. The PCR products were visualized using agarose gel electrophoresis (Fig. 5). The pattern of expression of the αC helix (390 bp) was different in both cell lines (Fig. 5).

The bands were then isolated using a Zymoclean Gel DNA Recovery kit and sequenced using an ABI Prism sequencer. The analysis of the obtained DNA sequences were then compared to the normal EGFR sequence reported and is shown in Fig. 6. The obtained sequence for the EGFR present in the CALO cell line is shown in Fig. 6A. The presence of multiple deletions (positions 19, 20, 40, 78 and 79), one insertion (position 126), transversions (124, 157, 214 and 224) and transitions (215, 227, 228 and 254) was detected. Using the BLASTx program, the DNA sequence was translated into the putative amino acid sequence. The putative amino acid sequence of the EGFR present in the CALO cell line showed multiple differences as compared with that in the normal EGFR amino acid sequence. For example, deletions of amino acids (positions 3, 18, 19, 21-25 and 47-49), conservative changes (positions 4-6, 11, 16, 31, 33, 36, 44 and 46) and non-conservative changes (positions 2, 8, 10, 12-14, 17, 27-29, 32, 34, 35, 38, 39, 41, 42, 45, 51, 58, 77, 82, 93 and 94) (Fig. 6A-a).

With respect to the INBL cervical cancer cell line (Fig. 6B), the DNA sequence of the EGFR showed only three changes, such as a deletion (position 10), a transversion (position 64)

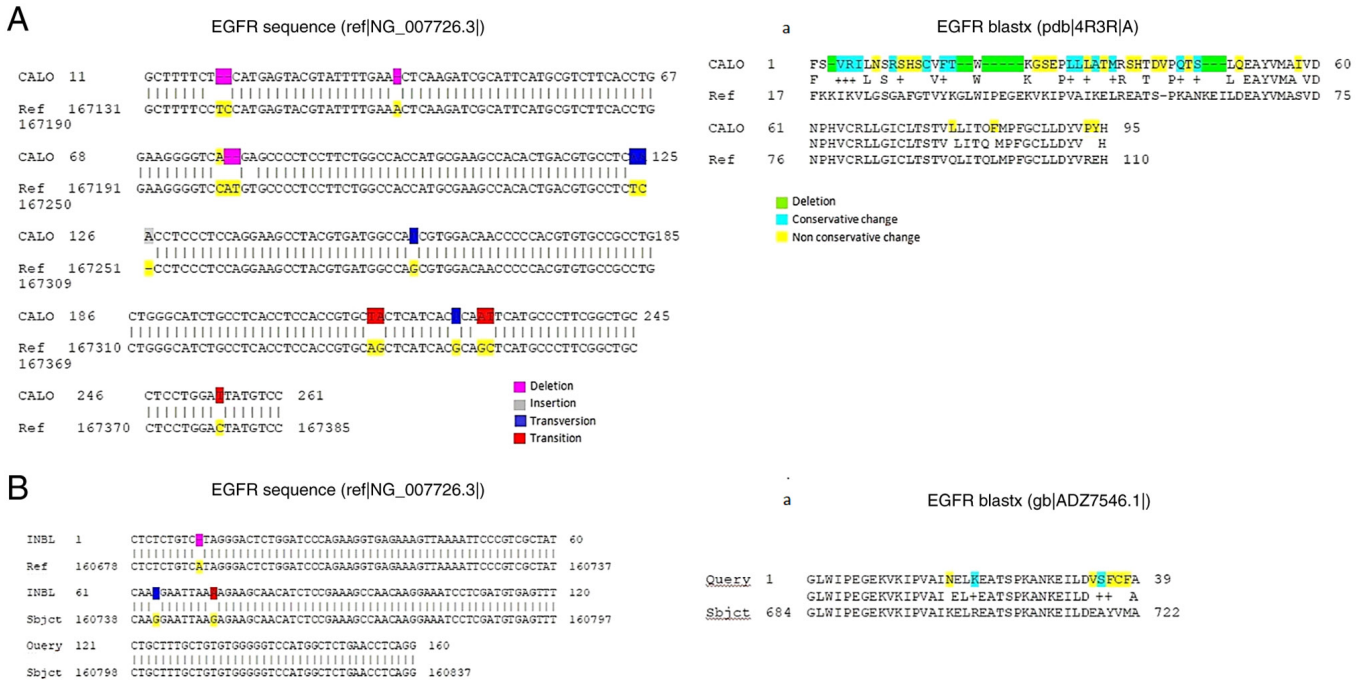

Figure 6. EGFR gene sequence. The EGFR sequence was identified from (A) CALO and (B) INBL cervical cancer cell lines using DNA sequencing. The sequence of the EGFR obtained from the CALO and INBL cervical cancer cell lines was compared with the EGFR reference sequence, RefSeqGene (LRG_304) on chromosome 7 (NG_007726.3). Mutation points are shown in yellow. The sequences obtained from the (A-a) CALO and (B-a) INBL cell lines were analyzed using BLASTx to obtain the putative amino acid sequences. Mutation points are shown in light blue.

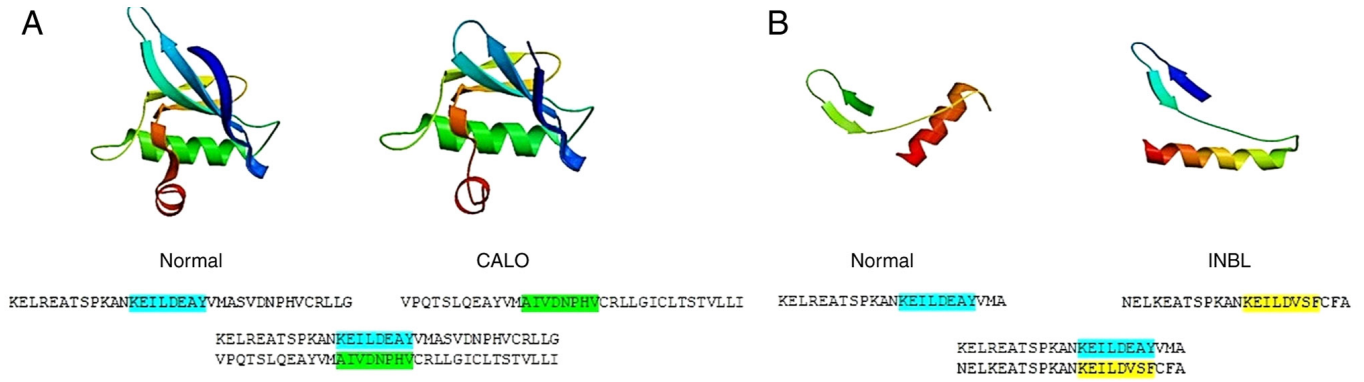

Figure 7. Model of the putative amino acid sequence for the helix αC domain from the EGFR present in the cervical cancer cells. Normal sequence is shown in light blue. The sequences obtained using BLASTx were modeled for the (A) CALO (green) and (B) INBL (yellow) cell lines.

and a transition (position 72). The translation of the DNA sequence into the putative amino acid sequence, using the BLASTx program, is shown in Fig. 6B-a. The comparison with the EGFR reference sequence indicated some differences: Non-conservative (positions 17, 34, 36, 37 and 38) and conservative differences (positions 20, and 36).

The DNA sequences were processed by BLASTx and the putative amino acid sequences were obtained. The results showed that in the CALO cervical cancer cell line the conserved glutamic acid residue at position 738 in the normal EGFR was replaced by asparagine, and in the INBL cell line, the glutamic acid residue was replaced by valine. Other residues found in the reference sequence of the helix αC in the normal EGFR were also different in the EGFR present in the cervical cancer cell lines. In the CALO cell line, a sequence of amino acids contained multiples changes. The most significant was the lack of the KEILDEAY motif in the CALO cell line. The sequence, AIVDNPHV, lacked tyrosine and other necessary residues (only conserved D) to form the interphase between the dimers. The INBL cell line contained the sequence, KEILDVSF, which is similar to the reference EGFR sequence; however, it included three different amino acids. Both cell lines contained the loss of the phosphorylated tyrosine present in the KEILDEAY motif (Fig. 7A and B). These putative amino acid sequences were modelled using the SWISS-MODEL Workspace and the 3D structure showed differences in the amino acid spatial arrangement, for example, in the CALO cell line, the β-sheet was shorter and the torsion was slightly lower; in the INBL cell line the most evident change was the torsion, which was bigger (Fig. 7A and B).

Discussion

The understanding of cancer biology has led to the identification of numerous receptors with tyrosine kinase activity (RTK) (12,24) involved in malignant transformation. The EGFR is the most common RTK that is mutated and overexpressed in epithelial cancers (25-28). Our previous research showed that the EGFR was present in the CALO and INBL cervical cancer cell lines, but was not phosphorylated (19); therefore, we hypothesized that it was a dead kinase. To investigate the hypothesis, the present study was performed to determine if it had null catalytic activity due to a mutation in the catalytic domain. The amplification of the EGFR gene is common in cancer and mutations in the EGFR tyrosine kinase domain have been demonstrated to occur in cervical cancer; these mutations are mainly somatic (13-17). The present study provides evidence for *in vitro* catalytic activity of this molecule, despite the presence of some mutations in the catalytic domain in both cell lines. In addition, it was found that the immunoprecipitated EGFR, from the activated CALO and INBL cell lines was not phosphorylated compared with that in the HeLa and CasKi cell lines (Fig. 1). This is unusual as typically this receptor is hyperphosphorylated. Our previous research demonstrated that the CALO and INBL cell lines expressed EGFR, HER2 (19) and JAK3 (24), and only the EGFR was found not to be constitutively phosphorylated. However, when the EGFR was isolated and analyzed using an *in vitro* kinase assay (Fig. 2), it was found that the receptor had catalytic and autophosphorylating activity. It could be possible that in the CALO and INBL cervical cancer cell lines, the ERRFI1 molecule could block the autocatalytic activity of EGFR, which is responsible for the inhibition observed (29,30). In the present study, a differential behavior of the catalytic activity of the receptor was found. It is proposed that when the isolated receptor is washed under stringent conditions, non-covalent interactions are broken. Therefore, the regulator molecule might be separated, enabling the catalytic activity of EGFR.

On the other hand, EGFR is part of the ErbB family of tyrosine kinases receptors. The activation of EGFR by its ligand leads to autophosphorylation; this change initiates a cascade of downstream signaling pathways regulating cellular proliferation, differentiation and survival (8,26,30). With respect to HER3, this kinase lacks several residues that are important for catalysis. Since the activation of EGFR family members is switched by an allosteric interaction between the kinase domain in an asymmetric kinase domain dimer, HER3 is specialized to serve as an activator of other EGFR family members (8-11). The helix αC is an essential element in kinase regulation. The conformation of this domain defines an inactive conformation that is characteristic of numerous kinases, such as CDKs (31), Src kinases (32), Zap70 (33) and EGFR (34). In the active conformation, the helix αC has a conserved glutamic acid residue (position 738 in EGFR), which is able to form a salt bridge with a conserved lysine (position 721 in EGFR), which coordinates ATP (8). In HER3, the glutamic acid 738 residue is replaced by histidine, an amino acid with a positive charge. It has been reported that the recombinant HER3 does not have a detectable kinase activity (10) or has minimal activity when immunoprecipitated (11,35). Due to these characteristics, HER3 has been designated as a pseudokinase (8,9,36). Similarly, in the CALO cervical cancer cell line, in the putative sequence of helix αC, in the catalytic domain, the conserved glutamic acid 738 residue is replaced by asparagine, and in the INBL cell line it is replaced by valine. Therefore, both amino acids lack a negative charge and cannot form a saline bridge with the conserved residue lysine 721. There were also differences in the helix αC from the isolated EGFR in comparison to the EGFR reference sequence. In the CALO cell line, one notable difference was found; the lack of the KEILDEAY motif, which changed to AIVDNPHV. It lacked the tyrosine residue and other residues required to form the interphase between the dimers. Both cell lines lacked the tyrosine residue, that can be phosphorylated, present in the KEILDEAY motif; therefore, the activity of the receptor may change.

The activation of the EGFR family involves the formation of asymmetric dimers (homo- or heterodimers) between their kinase domains, in which one kinase domain (activator) acts as an allosteric activator of the other (receiver) (9,37). In this mechanism, the helix αC plays an essential role in the EGFR family by forming part of a docking site on the receiver domain for the activator kinase domain (9). This activation mechanism of the EGFR family resembles the activator kinase when it plays a similar role to that of the activation of a cyclin-dependent kinase (38).

With respect to HER3, the kinase domain lacks several residues that are necessary for catalysis. The activation of the EGFR family members is initiated by an allosteric interaction between the kinase domain in an asymmetric dimer. Thus, HER3 may have acquired a specialization to serve as an activator of the EGFR family members (9). Similarly, the EGFR present in the CALO and INBL cervical cancer cell lines did not show catalytic activity in the presence of other molecules in the cellular environment in the present study. In addition, it contained differences in the amino acids located in the catalytic domain (Figs. 6 and 7). Thus, it is hypothesized these changes in the amino acid sequence and the spatial arrangement may change the EGFR conformation and confer a different activity in these cell lines, such as an activator of asymmetric dimers emulating the function of HER3.

The weak kinase activity of EGFR in the CALO and INBL cervical cancer cell lines may resemble the ability of the HER3 kinase to drive resistance to agents which inhibit other family members, for example EGFR or HER2 (39). Therefore, the EGFR without kinase activity in the cellular environment in both the CALO and INBL cell lines may serve to activate HER2 (which is constitutively active) (19) and, similar to HER3, also plays a critical role in the ability of HER2 to escape growth inhibition by some inhibitors, such as lapatinib (40). It was demonstrated that the EGFR protein does not interact with HER2 in the CALO and INBL cell lines; however, it is necessary to determine if this interaction is possible in other cervical cell lines, such as HeLa and CasKi, to have a complete understanding of this function of EGFR protein. In addition, further studies are being conducted to prove the interaction between the EGFR protein and ERRF1 and its participation in cervical carcinogenesis, as this molecule has an intrinsic oncogenic potential.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AVM and ISC were involved in the conception and design of the study. AVM, RBR, OZC and VDM performed the experiments. AVM, RBR and OZC analyzed the data. AVM, RBR, OZC, VDM, AGH, BWS and ISC interpreted the results of the experiments. AVM and VDM performed analysis of the sequencing data. AVM, RBR and OZC prepared the figures. AVM, VDM, AGH and ISC drafted the manuscript. AVM, VDM, AGH, BWS and ISC edited and revised the manuscript. AVM, VDM, AGH, BWS and ISC approve the final version of the manuscript. All authors have read and approved the final manuscript. AVM, OZC and ISC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, *et al*: A review of human carcinogens-Part B: Biological agents. Lancet Oncol 10: 321-322, 2009.
3. Zur Hausen H: Papillomaviruses and cancer: From basic studies to clinical application. Nat Rev Cancer 2: 342-350, 2002.
4. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsaque X, Shah KV, Snijders PJ and Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group: Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348: 518-527, 2003.
5. Soonthornthum T, Arias-Pulido H, Joste N, Lomo L, Muller C, Rutledge T and Verschraegen C: Epidermal growth factor receptor as a biomarker for cervical cancer. Ann Oncol 22: 2166-2178, 2011.
6. Stöppler MC, Straight SW, Tsao G, Schlegel R and Mccance DJ: The E5 gene of HPV16 enhances keratinocyte immortalization by full-length DNA. Virology 223: 251-254, 1996.
7. Tomakidi P, Cheng H, Kohl A, Komposch G and Alonso A: Modulation of the epidermal growth factor receptor by the human papillomavirus type 16 E5 protein in raft cultures of human keratinocytes. Eur J Cell Biol 79: 407-412, 2000.
8. Jura N, Shan Y, Cao X, Shaw DE and Kuriyan J: Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3. Proc Natl Acad Sci USA 106: 21608-21613, 2009.
9. Sierke SL, Cheng K, Kim HH and Koland JG: Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. Biochem J 322: 757-763, 1997.
10. Wallasch C, Weiss FU, Niederfellner G, Jallal BA, Issing W and Ullrich A: Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J 14: 4267-4275, 1995.
11. Shi F, Telesco SE, Liu Y, Radhakrishnan R and Lemmon MA: ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci USA 107: 7692-7697, 2010.
12. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, *et al*: Activating mutations in the epidermal growth factor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129-2139, 2004.
13. Iida K, Nakayama K, Rahman MT, Rahman M, Ishikawa M, Katagiri A, Yeasmin S, Otsuki Y, Kobayashi H, Nakayama S and Miyazaki K: EGFR gene amplification is related to adverse clinical outcomes in cervical squamous cell carcinoma, making the EGFR pathway a novel therapeutic target. Br J Cancer 105: 420-427, 2011.
14. Wolber L, Hess S, Bohlken H, Tennstedt P, Eulenburg C, Simon R, Gieseking F, Jaenicke F, Mahner S and Choschzinck M: EGFR gene copy number increase in vulvar carcinoma is linked with poor clinical outcome. J Clin Pathol 65: 133-139, 2012.
15. Li Q, Tang Y, Cheng X, Ji J, Zhang J and Zhou X: EGFR protein expression and gene amplification in squamous intraepithelial lesions and squamous cell carcinomas of the cervix. Int J Clin Exp Pathol 7: 733-741, 2014.
16. Conesa-Zamora P, Torres-Moreno D, Isaac MA and Pérez-Guillermo M: Gene amplification and immunohistochemical expression of ERBB2 and EGFR in cervical carcinogenesis. Correlation with cell-cycle markers and HPV presence. Exp Mol Pathol 95: 151-155, 2013.
17. Li Q, Cheng X, Ji J, Zhang J and Zhou X: Gene amplification of EGFR and its clinical significance in various cervical (lesions) lesions using cytology and FISH. Int J Clin Exp Pathol 7: 2477-2483, 2014.
18. Arias-Pulido H, Joste N, Chavez A, Muller CY, Dai D, Smith HO and Verschraegen CF: Absence of epidermal growth factor receptor mutations in cervical cancer. Int J Gynecol Cancer 18: 749-754, 2008.
19. Soto-Cruz I, Rangel-Corona R, Valle-Mendiola A, Moreno-Morales X, Santiago-Pérez R, Weiss-Steider B and Cáceres-Cortés JR: The tyrphostin B42 inhibits cell proliferation and HER-2 autophosphorylation in cervical carcinoma cell lines. Cancer Invest 26: 136-144, 2008.
20. Caceres-Cortes JR, Alvarado-Moreno JA, Waga K, Rangel-Corona R, Monroy-Garcia A, Rocha-Zavaleta L, Urdiales-Ramos J, Weiss-Steider B, Haman A, Hugo P, *et al*: Implication of tyrosine kinase receptor and steel factor in cell density-dependent growth in cervical cancers and leukemias. Cancer Res 61: 6281-6290, 2001.
21. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L and Schwede T: SWISS-MODEL: Modeling protein tertiary and quaternary structures using evolutionary information. Nucleic Acids Res 42: W252-W258, 2014.
22. Arnold K, Bordoli L, Kopp J and Schwede T: The SWISS-MODEL workspace: A web-based environment for protein structure homology modeling. Bioinformatics 22: 195-201, 2006.
23. Benkert P, Biasini M and Schwede T: Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 27: 343-350, 2011.

24. Valle-Mendiola A, Weiss-Steider B, Rocha-Zavaleta L and Soto-Cruz I: IL-2 enhances cervical cancer cells proliferation and JAK3/STAT5 phosphorylation at low doses, while at high doses IL-2 has opposite effects. Cancer Invest 32: 115-125, 2014.
25. Yarden Y and Pines G: The ERBB network: At last, cancer therapy meets systems biology. Nat Rev Cancer 12: 553-563, 2012.
26. London M and Gallo E: Epidermal growth factor receptor (EGFR) involvement in epitelial-derived cancers and its current antibody-based immunotherapies. Cell Biol Int 44: 1267-1282, 2020.
27. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, Majem M, López-Vivanco G, Isla D, Provencio M, *et al*: Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 361: 958-977, 2009.
28. Ayati A, Moghimi S, Salarinejad S, Safavi M, Pouramiri B and Foroumadi A: A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an efficient approach in cancer targeted therapy. Bioorg Chem 99: 103811, 2020.
29. Anastasi S, Baietti MF, Frosi Y, Alema S and Segatto O: The evolutionarily conserved EBR module of RALT/MIG6 mediates suppression of the EGFR catalytic activity. Oncogene 26: 7833-7846, 2007.
30. Singh D, Attri BK, Gill RK and Bariwal J: Review on EGFR inhibitors: Critical update. Mini Rev Med Chem 16: 1134-1166, 2016.
31. De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO and Kim SH: Crystal structure of cyclin-dependent kinase 2. Nature 363: 595-602, 1993.
32. Xu W, Doshi A, Lei M, Eck MJ and Harrison SC: Crystal structure of c-Src reveal features of its autoinhibitory mechanism. Mol Cell 3: 629-638, 1999.
33. Deindl S, Kadlecek TA, Brdicka T, Cao X, Weiss A and Kuriyan J: Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell 129: 735-746, 2007.
34. Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Ellis B, Pennisi C, Horne E, Lackey K, *et al*: A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationship among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. Cancer Res 64: 6652-6659, 2004.
35. Manning G, Whyte DB, Martinez R, Hunter T and Sudarsanam S: The protein kinase complement of the human genome. Science 298: 1912-1934, 2002.
36. Guy PM, Platko JV, Cantley LC, Cerione RA and Carraway KL III: Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci USA 91: 8132-8136, 1994.
37. Zhang X, Gureasko J, Shen K, Cole PA and Kuriyan J: An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125: 1137-1149, 2006.
38. Jeffrey PD, Russo AA, Polyak K, Gibbs E, Hurwitz J, Massague J and Pavletich NP: Mechanism of CDK activation revealed by structure of a cyclinA-CDK2 complex. Nature 376: 313-320, 1995.
39. Baselga J and Swain SM: Novel anticancer targets: Revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer 9: 463-475, 2009.
40. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM and Moasser MM: Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 445: 437-441, 2007.