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

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Received September 15, 2021; Accepted November 26, 2021

DOI: 10.3892/ol.2022.13191

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.

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% CO₂.

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 phenylmethansulfonylfluoride, 1 mM NaF, 1% aprotinin, 1 µM leupeptin, 1 µM pepstatin and 100 µM Na₃VO₄] for 15 min. The lysates were then 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).

For 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, 3x10⁶ 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'-CGT AAA CGT CCC TGT GCT AGG-3' and reverse, 5'-GTC GGG TCA ACG CTA GGC TG-3' (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH forward was 5'-GGGACGCTTGTGTCATG-3' and reverse was 5'-GTCGGGTCACACGGTCTGAGGC-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
Our previous research demonstrated that the EGFR receptor isolated from the CALO and INBL cervical cancer 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, 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).

**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 demonstrated that the EGFR was not phosphorylated in the CALO and INBL cervical cancer 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 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...
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. 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)
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 $\alpha$C 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 $\beta$-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 ERRFI1 and its participation in cervical carcinogenesis, as this molecule has an intrinsic oncogenic potential.
Acknowledgements

The authors would like to thank Mr. José Chavarría-Mancilla (Laboratory of Molecular Oncology, FES Zaragoza, National University of Mexico, Mexico City, Mexico) for technical assistance.

Funding

This study was supported by grants from Programa de Apoyos a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) (grant nos. IN221512 and IN222218) from the Dirección General de Asuntos del Personal Académico (DGAPA) Universidad Nacional Autónoma de México (UNAM).

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. 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.

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