Original Article

HeLa Cell Line Xenograft Tumor as a Suitable Cervical Cancer Model: Growth Kinetic Characterization and Immunohistochemistry Array

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Abstract

Background: Cervical cancer is the seventh most common malignancy in both genders combined and the third most common cancer in women. Despite significant progress in treatments, cervical cancer is not completely curable. Therefore, further research is necessary in this area. Animal models are one of the most practical tools in the field of cancer research. The present study aimed to characterize the growth behavior and surface markers of HeLa cells after heterotopic and systemic inoculation to athymic nude mice.

Methods: Ten 6-week old female athymic *C57BL/6* nude mice were used in this study. HeLa cells were inoculated into the flank or tail vein. The tumor volume was calculated and growth curves were drawn. Tumor-bearing mice were sacrificed and the lesions obtained after harvesting were analyzed in a pathology lab. Subsequently, one slide per tumor was stained with hematoxylin and eosin (H&E) and other slides were stained immunohistochemically by cytokeratins (CK), vimentin, P53, CD34, and Ki-67.

Results: Tumor take rate, mean doubling time and latency period were 94.4%, 5.29 ± 3.57 days and 15.27 days, respectively. H&E results revealed highly malignant hyperchromatin epithelial cells. Immunohistochemical examination of the heterotopic tumors indicated greater expression of CK and less expression of vimentin compared to the metastatic ones. Sixty percent of cells were P53-positive and more than 80% were Ki-67-positive. CD34 expression indicated the intensity of angiogenesis in tumor.

Conclusion: This study represents a comprehensive description of a HeLa xenograft model for *in vivo* investigations, enabling researchers to assess new treatments for cervical cancer.

Keywords: HeLa cells, immunohistochemistry, neoplasm transplantation, uterine cervical neoplasm

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Introduction

Cervical cancer is the seventh most common malignancy in both sexes combined and the third most common cancer in women.[1,2] Epidemiological studies in Iran describe cervical cancer as one of the five common malignancies in females.[3] Overall, human papillomaviruses (HPV), particularly HPV-16 and HPV-18, inadequate screening, multiple sex partners, young age at intercourse, male sexual behavior, and tobacco use are commonly known crucial risk factors of cervical neoplasm.[4–7]

Treatment usually consists of surgery in early stages (including *local excision*), and chemotherapy and/or radiotherapy in more advanced stages of the disease.[8] Survival improves when radiotherapy is combined with cisplatin-based chemotherapy.[9] Despite significant progress in its treatment, cervical cancer is not completely curable. Therefore, it is necessary to conduct further research in this area.[10] Development of novel therapeutic approaches requires appropriate research tools. Animal models are one of the most practical tools in the field of cancer research. Experience shows that diseases which benefit from appropriate models also have proper therapies.[11]

At present, several cervical cancer animal models exist, including carcinogenic, syngeneic and xenograft models. Risk of contact with carcinogenic agents, long tumor induction time and tedious evaluation parameters are the demerits of carcinogenic models.

Syngeneic tumor models, a mouse or rat tumor growing in mice or rats of the strain in which the tumor originated, lack several important features of human tumors. For example, they are usually derived from homozygous inbred mice, and thus lack the genetic complexity of human tumors. In addition, due to species-specific differences, they may not bear the same constellation of mutations observed in human patients.[12]

Human tumor xenograft models established in immunodeficient mice using human neoplastic cell lines are considered to be the most clinically relevant models. These models are the workhorse for anticancer drug discovery and development programs.[13]

To date, several cervix carcinoma xenograft models have been developed with different cell lines.[14] The HeLa cell line is the first continuously cultured human neoplastic cell line, derived from the cervical carcinoma of a woman in 1965.[15] Despite the presence of a large number of studies dealing with the establishment of the xenograft model of HeLa cells and screening of different

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anticancer agents on it, integrated data is not available on the characteristics of this model.

The present study aimed to characterize growth, metastatic behaviors and surface markers of HeLa cells after heterotopic and systemic inoculation to athymic nude mice.

Materials and methods

Ten 6-week old female athymic *C57BL/6* nude mice were purchased from Omid Institute for Advanced Biomodels (Iran). Autoclaved water and food were available *ad libitum* to the mice. The experiment was conducted in compliance with ethical principles approved by international animal care and use committees. The HeLa cell line was obtained from the National Cell Bank of Iran, Pasteur Institute (NCBI), and cultured in RPMI 1640 containing 10 percent fetal bovine serum (FBS). A total of 5×10^6 cells were inoculated subcutaneously in 200 µL of serum–free medium into the flank of the animals bilaterally. Tumor growth was measured twice a week. The volume of tumors was calculated using a standard formula (length × width^2 × 0.52) and growth curves were drawn [16].

Two months later, the mice were sacrificed by CO_2 inhalation and harvested tumors were fixed in 10% buffered formalin and analyzed in the pathology lab.

HeLa cells were also inoculated into the tail vein (1×10^6 cells) in order to establish a metastatic model. One month later, the metastatic animal model was sacrificed. To examine metastatic foci, the lungs were removed, washed in water, and placed in Bouin's fixative. Then, metastatic tissues were examined histopathologically.

For pathologic examination of both heterotopic lesions, one slide per tumor was stained with hematoxylin and eosin (H&E) and another slide was stained by immunohistochemistry (IHC) with cytokeratins (CK) and vimentin (to confirm the ectodermal or mesenchymal origin of the tumor), P53 (to evaluate TP53 mutation), Ki-67 (to assess tumor proliferation), CD34 (to evaluate tumor angiogenesis using microvessels density [MVD]) tumor marker antibodies (DAKO Company) for each tumor. Metastatic lesions were examined by H&E staining and CK and vimentin markers.

For quality control of IHC assay, human fibrosarcoma and human squamous cell carcinoma paraffin blocks were used as external positive controls for vimentin and CK markers, respectively. Negative controls were performed by omission of the secondary antibodies of vimentin, CK, Ki-67, p53 and CD34 markers.

Results

After subcutaneous inoculation of a cervical cancer cell line (HeLa cell line) to 9 female mice, 17 tumors were formed (Figure 1A) and tumor cells did not grow in only one inoculation site. The growth curve of tumors is shown in Figure 1B. Tumor take rate, mean doubling time and latency period were 94.4%, 5.29 ± 3.57 days and 15.27 days, respectively.

H&E results of heterotopic tumors showed highly malignant hyperchromatin epithelial cells. Atypical mitosis and numerous nucleoli were also observed inside the cells. Numerous necrotic areas were illustrated, as well (Figure 2A). Due to the severity of malignancy, carcinoma was diagnosed as undifferentiated. Malignant mesenchymal-like cells within neoplastic epithelial cells were also detectable. IHC examination showed that CK (Figure 2B) and vimentin (Figure 2C) were strongly positive. Vimentin expression could be observed within malignant mesenchymal-like cells more frequently. More than 80% of cells were Ki-67-positive (Figure 2D) and sixty percent of them were P53-positive (Figure 2E). CD34 expression (Figure 2F) indicated the intensity of angiogenesis in tumor.

Metastasis was established in lung tissue one month after intravenous inoculation of the cells. Metastatic lesions were observed grossly after fixation in Bouin's fixative (Figure 3A). Histopathological examination of the lungs illustrated homing of malignant cells which were histomorphologically similar to those observed in the heterotopic ones (Figure 3B). IHC results of the metastatic lung foci indicated less expression of CK (Figure 3C) and greater expression of vimentin (Figure 3D) compared to the heterotopic tumors; a pattern implicating epithelial-mesenchymal transition (EMT).

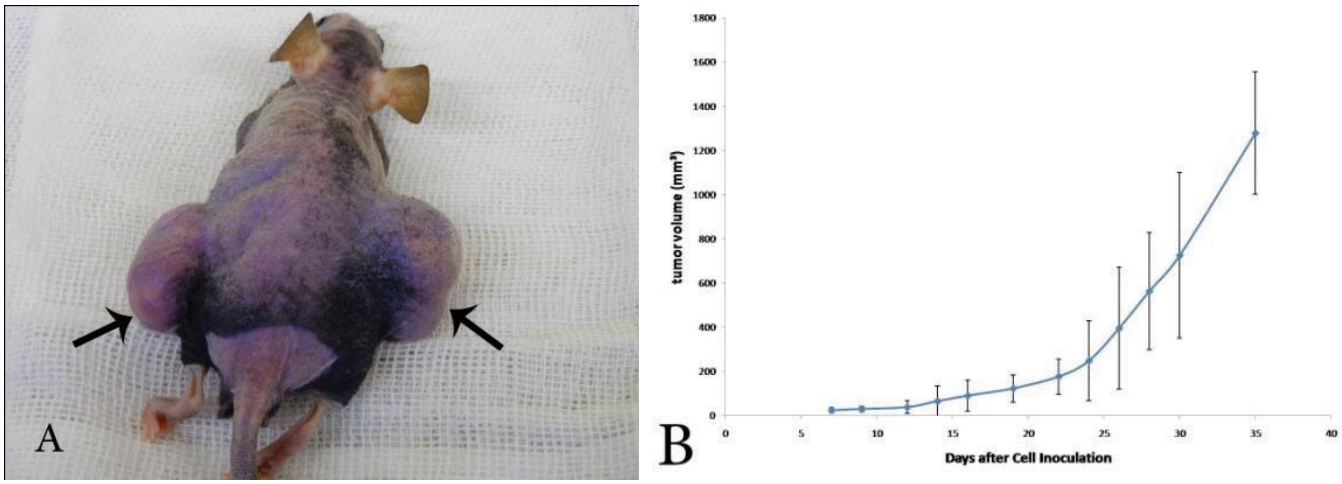

Figure 1. A) Heterotopic HeLa xenograft cervical cancer: 5×10^6 cells were subcutaneously inoculated into the flank of the animal bilaterally; **B)** Growth curve of cervix tumors during 30 days after inoculation of HeLa cells. Error bars represent standard deviation.

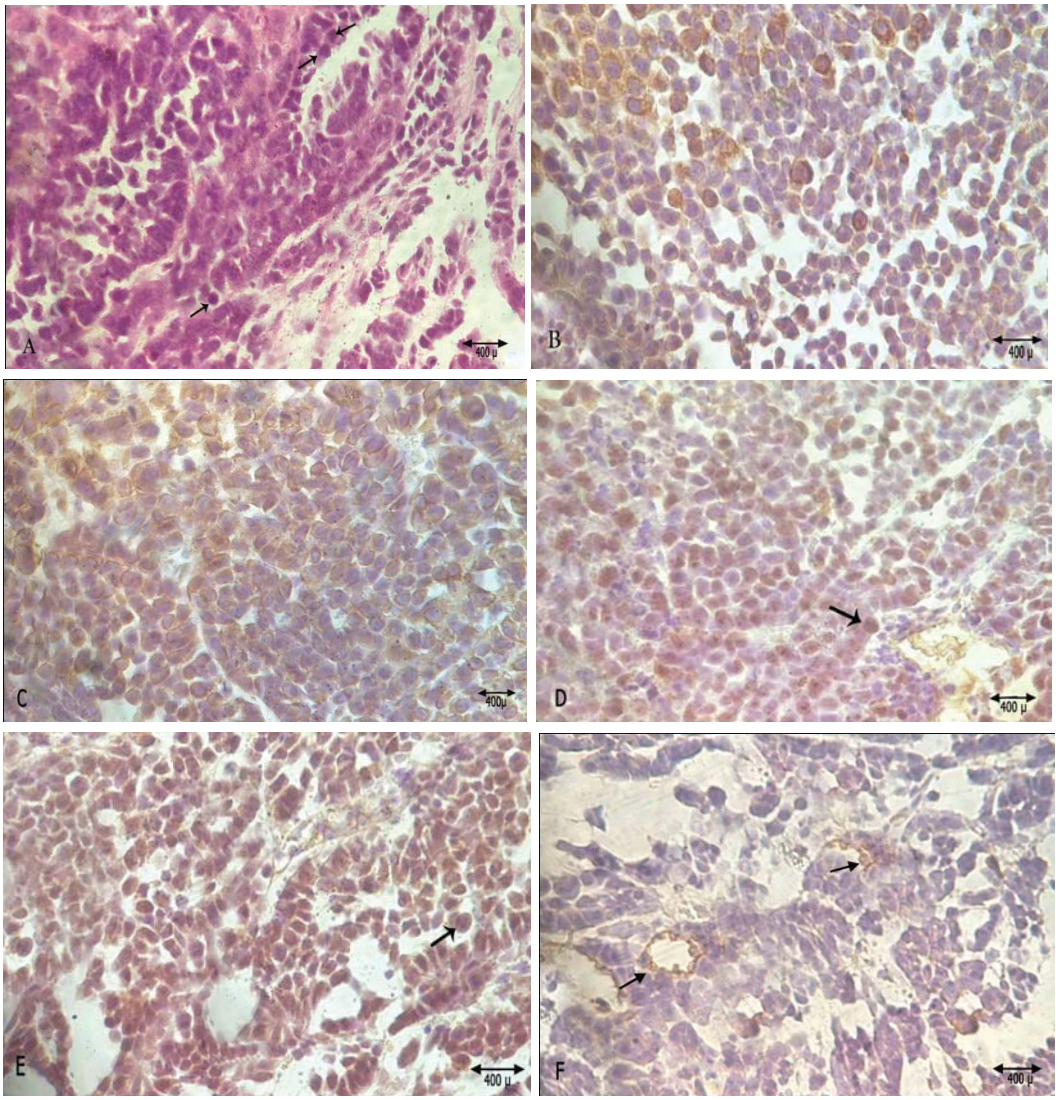

Figure 2. Photomicrographs of xenograft cervical tumor of HeLa cell line. **A)** H&E staining. Highly malignant cells with atypical mitosis and pleomorphism are observable. Arrows shows mitotic stages. **B)** IHC staining using CK marker. Immunoreactivity and CK positivity are detectable in malignant cells' membrane. CK positivity rate and intensity are high. **C)** IHC staining using vimentin marker. This figure shows vimentin immunoreactivity. Mild to severe staining of membrane is detectable. **D)** IHC staining using Ki-67 marker. Immunoreactive nuclei indicate high proliferation in the tumor xenograft model. The arrow shows one immunoreactive nucleus. **E)** IHC staining using P53 marker. High rate of Immunoreactive nuclei approve mutation in TP53 gene. The arrow shows one immunoreactive nucleus. **F)** IHC staining using CD34 marker. The arrows show Immunoreactive endothelial cells as brown color lumens. This slide was evaluated by MVD- CD34 method.

Discussion

This study aimed to characterize comprehensively the xenograft cervical carcinoma model of the HeLa cell line. Pathological and kinetic growth properties were characterized. Moreover, this study was successful in establishing a metastatic model for the HeLa cell line. EMT, high angiogenesis and undifferentiation were also significant within tumor cells.

The HeLa cell line is the oldest, most widely distributed, permanent human cell line.[17] The line was derived from cervical cancer cells taken on February 8, 1951 from Henrietta Lacks, a patient who eventually died of her cancer.[15] The xenograft model of this cell line was established in 1976[18] and characterized in 1980 by Hisashi. In their study, the success rate of transplanting HeLa cell tumor into nude mice was always 100%. The latent period after inoculation of the cultured cell suspension was relatively short and lasted approximately one week after injection. The present study showed approximately the same take rate (94.4%), whereas latency periods were significantly different which may be caused by inoculation of smaller number of cells in our study.

Several metastatic models have been established for cervical cancer. Metastatic models of cervical cancer were represented using CaSki, ME-180, and SiHa cell lines and IP inoculation by Cairns in 2004.[19] Orthotopic xenograft of the ME-180 cell line also results in metastasis, which has been reported in two separate studies by Chaudary in 2011and 2013.[20,21] Yet, no metastatic model is available for the HeLa cell line. This study was able to successfully create a metastatic model by injection of cells via tail vein.

Microscopic data from this study demonstrated the validity of the model. Given the short doubling time of HeLa xenograft models, all nuclei features of a highly malignant cell can be observed. In our study, p53 was positive, whereas that of the primary tumor was also positive. Active nuclei and a high proliferation index

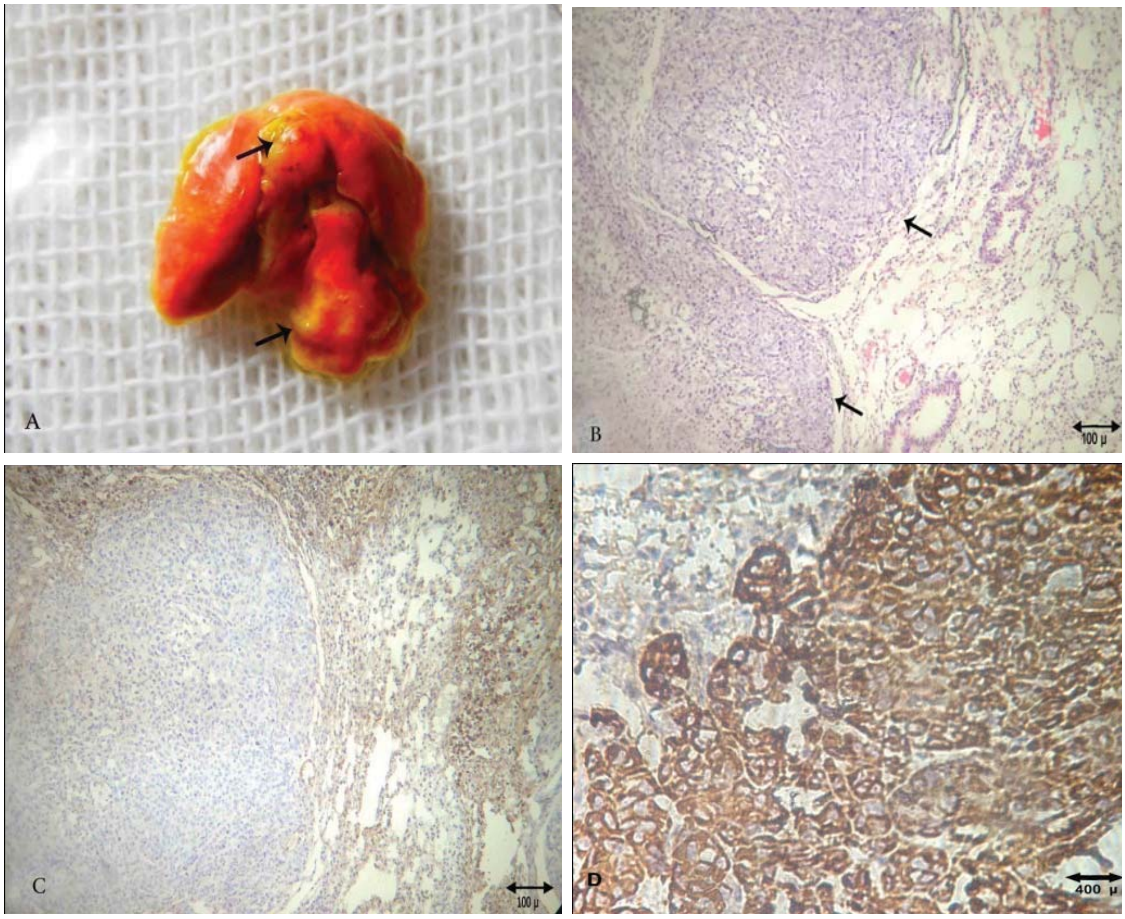

Figure 3. A) Metastatic lung: Approximately 1×10^6 cells were inoculated into the tail vein. Arrows indicate to the tumoral lesions. **B)** H&E staining. A mass of malignant metastatic cells is detectable within lung parenchyma. Arrows indicate borders between normal and metastatic tissues. **C)** IHC staining of metastatic lung using CK marker. CK shows mild to moderate immunoreactivity and is weakly positive. **D)** IHC staining of metastatic lung using vimentin marker. Vimentin is strongly positive showing EMT in metastatic area of HeLa cells.

(Ki-67) indicate a high level of metabolism in malignant cells. We have reported high rates of tumor angiogenesis. Any high metabolic activity in tumor cells induces hypoxia, which in turn induces the HIF-1 alpha gene and thus begins the phase of angiogenesis with new blood vessels formed.[22] Newly formed micro vessels in this study were labeled by CD34 marker and high MVD in tumors may be important in terms of the therapeutic value.[23] With inhibition of angiogenesis using anti-angiogenic drugs, we will be able to investigate targeted therapy in cervical cancer at the pre-clinical phase. CK marker in tumors shows their epithelial origin and vimentin confirms the mesenchymal origin of the tumor cells. In our study, as expected, CK was strongly positive, whereas expression of the vimentin marker was unexpected. In the past decade, EMT in cancers has received much attention. Epithelial cancer cells change their phenotypic and genetic characteristics in order to displace and invade. With the advent of genes such as matrix metalloproteinase-2 (MMP-2) and MMP-9, changes in some adhesive molecules cause epithelial cells to mimic the properties of mesenchymal stem cells and show phenotypic characteristics of fibroblast cells.[24] In this study, vimentin was expressed in heterotopic tumors and mesenchymal characteristics were emerging. Because cell nuclei are extremely active and angiogenesis and proliferation rate are also high, it may be concluded that these cells are ready for invasion and show EMT characteristics. Proving this hypothesis requires further tests. If confirmed, this model can be used for EMT studies.

Another finding of our study is greater expression of vimentin and less expression of CK in metastatic lung lesions compared to the heterotopic model. Unlike the heterotopic tumors, the ratio of CK to vimentin expression in the metastatic lesions is low. So, we can conclude that the EMT can be considered as a pattern of metastasis.

Lack of data from an orthotopic model can be considered as one of the limitations of this study. Therefore, further investigations are recommended to establish and characterize such a model for the HeLa cell line.

This study represents a comprehensive model of HeLa xenograft for *in vivo* investigation that enables researchers to assess new treatments for cervical cancer.

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