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Fatality Increases Esophageal Squamous Cell Arcinoma: Growth and Invasion via the AMPK-YAP Pathway

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Background:

Although the precise mechanisms are unknown, obesity may impact the growth and progression of esophageal squamous cell carcinoma (ESCC) as well as raise the risk of the disease. The aim of the research was to investigate the influence of obesity on the growth and progression of ESCC through in vitro cell assays and in vivo trials. TE-1 cells were injected into diet-induced obese and lean nude mice, and the mice were observed for four weeks. The levels of visfatin, leptin, insulin, and serum glucose were measured. Nude mouse sera were acquired and used to cultivate TE-1. RT-PCR, Western blotting, MTT, and migration and invasion tests were utilized to examineobesity's endocrine effects on TE-1 gene expression, cell migration, invasion, and proliferation. In addition to having larger tumor xenografts than lean animals, obese nude mice also had fatty livers, hyperglycemia, hyperinsulinemia, and higher levels of leptin and visfatin in their sera. In the subcutaneous tumor xenograft paradigm, obese nude mice had more aggressive tumors than lean ones. The weight of the tumor showed a positive correlation with serum glucose, HOMA-IR, visfatin, leptin, and mouse body weight as well as liver weight. An considerable increase in TE-1 cell migration, proliferation, andinvasion that affected target genes by endocrine pathways. In the cells cultured with conditioned media and xenograft tumor from the obese group, the expression of AMPK and p-AMPK protein decreased significantly ($P < 0:05$); MMP9, total YAP, p-YAP, and nonphosphorylated YAP protein increased significantly ($P < 0:05$); the mRNA expression of AMPK decreased significantly ($P < 0:05$); YAP and MMP9 mRNA expression increased significantly ($P < 0:05$) in the cells exposed to conditioned media from the obese group. In summary, changes in the adipokine milieu and metabolites associated with obesity may stimulate the growth of ESCC cells in vivo, impact their migration, invasion, and proliferation in vitro, and control the MMP9 and AMPK-YAP signaling pathway through a variety of intricate processes, including the endocrine effect.

1. Introduction

Globally, esophageal carcinoma (EC) is among the most prevalent gastric cancers. Over 95% of EC is caused by esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is the primary cause of cancer-related deaths and is more common in South America, Africa, and Asia [1].China death rate [2]. The majority of patients with ESCC are detected in the mid-dle or advanced stage, and the overall 5-year survival rate is only 10%, indicating a poor prognosis and significant fatality rate [3].Therefore, it is essential to investigate the mechanisms behind ESCC growth and development in order to find new therapeutic targets for the urgently needed adjuvant treatment.A global health concern, the prevalence of obesity has doubled in over 70 nations since 1980 and has been steadily rising in the majority of them [4]. It has been demonstrated that obesity increases the risk of acquiring certain malignancies, such as breast cancer, cardia gastric cancer, and EAC [5-7]. There is a complex link between obesity and ESCC, and there is an obesity paradox in cancer.Sity may encourage the growth of cancer, but it does not correspond with the patients' overall survival. This is particularly true for ESCC patients[5, 8], and agreement is difficult to come by. However, the prevailing view is that there may be a link between obesity—particularly abdominal obesity—and a higher risk of ESCC [9, 10].Obesity-related dysregulated metabolism, such as insulin resistance, dyslipidemia, and hyperglycemia, may result in the following: HindawiJournal of Immunology ResearchVolume 2020, Article ID 6765474, further impact the genesis and growth of tumors. White adipose tissue (WAT) accumulation is another effect of obesity. The adipokines that WAT secretes are soluble mediators that play a dynamic role in regulating metabolism, inflammation, immunity, and soon after. The altered adipokine milieu of obesity also has an impact on exacerbating the development of diseases like diabetes, cardiovascular disease, and cancer [11, 12]. WAT functions as an active endocrine organ. Cancer cell survival and solid tumor growth may be influenced by adipokines like leptin and other growth hormones released in the backdrop of obesity.development of tumors [11]. Leptin is known to be a possible mediator of obesity-associated malignancies and has a favorable correlation with both adiposestorage and nutritional status.

It also plays a significant role in energy balance and hunger control [13]. Visfatin, a recently discovered adipokine mostly released by visceral fat, rises in correlation with obesity [14] and regulates the metabolism of fats and carbohydrates. Visfatin could potentially play a significant role as a mechanistic link in the network of variables controlling the formation of tumors associated with obesity [15].Indeed-associated protein (YAP) is a transcriptional coactivator that controls several biological processes and operates downstream of the Hippo signaling pathway. Human cancers, such as gastric cancer [17], oral squamous cell carcinoma [16], and esophageal squamous cell carcinoma [18], typically had dysregulation of the Hippo-YAP pathway. Between the cytoplasm and the nucleus, YAP shuttles and interacts with transcription factors, especially those belonging to the TEA domain (TEAD) family.then triggers the production of antiapoptotic and proproliferative genes [19]. YAP is phosphorylated and rendered inactive in the cytoplasm upon receipt of an extracellular signal from the upstream Hippo kinase that inhibits cell development. This is followed by ubiquitin-mediated proteasomal destruction.

In contrast, hypophosphorylated YAP translocates into the nucleus in response to a growth encouraging signal from the kinase, where it increases the production of target proproliferative and antiagenes involved in poptotic processes. The catalytic α-subunit and regulatory β- and γ-subunits make up the highly conserved serine/threonine protein kinase known as adenosine monophosphate-activated protein kinase (AMPK). A conserved threo-nine residue (Thr172) in the α-subunit was phosphorylated by upstream protein kinases, which activated AMPK [20].A crucial cellular energy sensor called AMPK is triggered by raisingAMP levels are inactive in obese states and coordinate cell growth with available energy. Cellular energy stress causes AMPK-dependent Lats activation, which in turn causes YAP phosphorylation and inhibition of YAP function.Furthermore, YAP S94, a residue necessary for interacting with TEAD, was directly phosphorylated by AMPK, breaking the YAP-TEAD connection [21]. Consequently, the AMPK-YAProute may be important in the formation and progression of obesity-related malignancies and connects cellular energy state, such as obesity, to tumorigenesis. Early lymphatic and hematopoietic dispersion, which is intimately linked to tumor invasion and metastasis, can be facilitated by EC [22].The extracellular matrix (ECM) must be proteolytically cleaved by matrix metalloproteinases (MMPs) in order for tumor invasion and metastasis to occur. MMP2 and MMP9 are significant members of the MMPs' gelatinase family, which breaks down type IV collagen.enhanced tumor metastasis, as a result of a basement membrane component [23, 24]. Specifically, increased MMP9 is associated with a worse prognosis in some malignant tumors, such as EAC and ESCC [25–27].There are currently no appropriate animal models for studying the mechanisms by which obesity impacts the growth and development of ESCCs, and very little basic research has been done in this area. Therefore, to investigate the effects of obesity on ESCC growth and development and the associated molecular mechanism, an in vivo animal model and in vitro cell experiments were used in the study.

2. Materials and Methods

Cell culture and animals. The Institutional Animal Care and Use Committee of Xi'an Jiaotong University approved the use of animals in experiments. Malenude mice, aged four to five weeks, were procured from Slac Laboratory Animal (Shanghai, China). They were then kept under standard conditions and split into two groups, each of which was given a high-fat diet consisting of 35.0% fat, 26.0% carbohydrate, and 26.0% protein, and a normal chow consisting of 4.3% fat, 67.3% carbohydrate, and 19.2% protein) [28] for eight weeks. Typical ESCC cell lines, TE-1, kyse150, and Eca109, were obtained from the experiment platform. either acquired from the cell bank of the Chinese Academy of Sciences in Shanghai, China, or from Xi'an Jiaotong University. Every cancer cell was grown in Roswell Park Memorial Institute-1640 (RPMI-1640) media (Cellgro, Herndon, VA), which was enhanced with streptomycin (100 mg/mL), 10% (v/v) heat-inactivated fetal bovine serum (Valley Biomedical, Winchester, VA), and penicil-lin (100 U/mL). The cells were kept in a 37°C humidified incubator with 5% CO2 supply. TE-1 was selected for additional testing based on the MMP9 protein expression.ESCC Model in Vivo. After eight weeks, mice on a normal diet were classified as "lean," while mice on a high-fat diet were classified as "obese" if their body weight exceeded the mean plus two times the standard deviation (SD) of these lean nude mice. The mice who were left on the high-fat diet were referred to as "nonobese."Every day, the mice were observed after receiving a subcutaneous inoculation of 2:0 x 106 TE-1 cells in the right flank.Selected naked mice were kept for an additional 4 weeks on either a normal or high-fat diet. Once a tumor became palpable, its volume was measured by measuring its length and width using calipers and calculating 1/2 (length × width 2). Following a four-week period of injected tumor cell growth, all animals were euthanized under anesthesia, and blood was extracted from the retroorbital venous plexus to measure adipokines or metabolites. The tumors were weighed, meticulously dissected, and then kept for additional examination. To check for metastases, a laparotomy was performed. The liver was then removed, weighed, and kept for oil red O staining.Assays for serum. Following the manufacturer's instructions, the serum levels of insulin, leptin, and visfatin were measured using an enzyme-linked immunosorbent assay (ELISA). The TE-1 cells were cultured in conditioned medium or RPMI-1640 with 0.5% MTT solution for an additional 4 hours.

Afterwards, 150 µL of DMSO was added to dissolve the MTT tetrazolium crystal, and their optical density was measured at 490 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA). Every trial was conducted three times.Tests for Invasion and Migration. Assays for migration and invasion were carried out in accordance with the guidelines to ascertain how TE-1 cells migrated and invaded after being exposed to conditioned medium. In each chamber, 5 × 104 TE-1 cells were added to the upper wells of either the invasion or migration chambers using a matrigel membrane. For a whole day, either serum-free RPMI-1640 or conditioned media were added to the wells under the chambers. Following the extraction of cells from the upper surface of the filters, the remaining cells on the lower surface were systematically counted using a microscope in ten distinct high-power fields (magnification, ×200). Studies on NA

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Expression. Using the TRIzol reagent (Invitrogen, San Diego, CA), total RNA was isolated from target cells after they were exposed to RPMI-1640 or conditioned medium for 24 hours. The cDNA was then reverse transcribed using an acDNA Synthesis Kit (Takara Biochemicals, Japan), according to the manufacturer's instructions. RT-PCR was used to measure the expression of AMPK and Yap mRNA (Takara Biochemicals, Japan). GAPDH was used to standardize transcript levels.In fairness, the reaction was carried out using an iCycler (Bio-Rad, Hercules, CA) with the subsequent thermal protocol: a 10-minute preheating phase, followed by 30-second intervals of 94.0°C, 57.0°C, and 72°C. NCBI Primer-BLAST was used to create the sequence for these primers (Table 1). Every trial was conducted three times for validation.

Western Blotting Examination. The impact of obesity on the growth and development of TE-1 was investigated by Western blotting, using the previously published protocol, using target cells exposed to conditioned medium or RPMI-1640 over a 24-hour period or extracts from target tumor tissues [29]. After extracting 25 µg of protein, it was separated using SDS-PAGE and electroblotted onto nitrocellulose membranes for Western blotting examination. Blots were incubated with HRP-conjugated secondary antibody for one hour at room temperature after being probed with suggested diluted primary antibodies for an overnight period at 4°C. Amersham Bioscience, Piscataway, New Jersey, USA, created an enhanced chemiluminescence detection technique for the membranes, resulting in Pierce chemiluminescence. The primary antibodies were obtained from Beijing Biosynthesis Biotechnology (China) and recognized AMPK, p-AMPK, YAP, p-YAP, and GAPDH. Analytical Statistics. The mean +/- SD was used to express the values. One-way analysis of variance (ANOVA) was used to examine statistical differences, and Dunnett's test was used to follow it. The Pearson test was used to estimate correlation analysis. P values less than 0.05 were regarded as statistically significant.

3. Results

MMP9 Protein Expression in ESCC Cell Lines. Examining the invasion of ESCC and MMP9 protein expression in common ESCC was one of the study's main goals.Analysis was done on cell lines such TE-1, kyse150, and Eca109.The study's findings showed that TE-1 expressed the least of all of them, hence it was selected as the main cell line for additional testing.Mouse Metabolic Alterations. After eight weeks, 15 mice given the regular diet were labeled as "lean," 14 of the 40 mice given the high-fat diet were designated as "obese," and the 26 mice that remained in the high-fat diet group were labeled as "nonobese." These obese mice were significantly heavier than lean mice at the time of vaccination (28:04 ± 0:53 versus 23:84 ± 1:86g, P < 0:001), but there was no difference between the two groups of mice (23:84 ± 1:86 versus 22:01 ± 2:32 g, P = 0:42).
Tumor Progression and Evolution. For a further four weeks, the standard or high-fat diet was given to the injected nude mice.During the course of the experiment, no metastasis was seen in any of these mice, and they were all still alive. 93.3% (14/15) of lean mice, 92.9% (13/14) of obese mice, and 88.5% (23/26) of nonobese animals all had tumor development.Over the course of four weeks, obese nude mice had larger and faster growing tumors than lean and nonobese nude mice (Figure 2). Mice's body weight, tumor weight, and liver weight were gathered at the conclusion of the animal experiment (Table 2). The weight of the tumor had a significantly positive association with the mice's body weight (r s = 0:57, P< 0:001). Figure 3 shows that the obese mice also had fatty livers, and the tumor weight showed a high positive connection (r s = 0:62, P < 0:001) with the mice's liver weight.

Both adipokines and metabolic parameters. Serum levels of insulin, glucose, leptin, and visfatin were measured, and the homeostatic model assessment insulin resistance (HOMA-IR) score—a proxy for insulin resistance—was gathered (Table 3). These fat, naked mice showed signs of altered metabolism, including insulin resistance, hyperglycemia, and hyperinsulinemia, as well as a greater serum level of leptin.and visfatin at the conclusion of the animal trial compared to lean mice. Tumor weight showed a positive correlation with glucose (r s = 0:50, P < 0:001), visfatin (r s = 0:49, P < 0:001), HOMA-IR (r s = 0:45, P = 0:001), lep-tin (r s = 0:34, P = 0:017), and HOMA-IR (r s = 0:45, P = 0:001), but not with insulin (r s = 0:26, P = 0:064)Expression of the MMP9, AMPK, and YAP proteins in tumor tissues. Western blotting was used to examine relevant genes regarding tumor growth and development in the xenograft tumors, despite the absence of local or peritoneal metastases in the mice. The tumors from obese nude mice expressed considerably less AMPK and p-AMPK and more MMP9, YAP, and p-YAP than those from lean mice (Figure 4). Subsequent examination revealed that the tumor from the obese group had more nonphosphorylated YAP in its active form (14:3 ± 0:3 versus 1:1 ± 0:4% GAPDH, P < 0:001).Obesity's effects on the migration, invasion, and proliferation of tumor cells. TE-1 cells were used in in vitro cell studies under conditioned conditions to identify the involvement of the endocrine effect from obesity. 95% RPMI-1640 and 5% sera (v/v) from lean or obese mice were included in the conditioned medium. The TE-1 cell growth was evaluated using the MTT test. These groups included RPMI-1640 as the control group, obese, and lean individuals. Cell proliferation measured by the MTT test was monitored for five days following treatment. After creating a cell growth curve, the findings showed that fat stimulated the proliferation of TE-1 cells.

A cell migration and invasion assay was carried out because individual cell migration and invasion is a crucial feature of cancer cells that can result in local or distant metastases. In the high-power fields (magnification, ×200), the number of moving cells in the obese group was significantly higher than that of the lean group (270:1 ± 19:8 versus 158:0 ± 21:6/field, P < 0:001, Figure 6). In the RMPI-1640 group, there was no cell movement. In the high-power fields, the number of invasive cells in the obese group was significantly higher than that of the lean group (234:0 ± 26:9 versus 112:4 ± 18:9/field, P < 0:001, Figure 7). The findings demonstrated that fat acting as a chemokine caused TE-1 cells to migrate throughBeing overweight AMPK-YAP and MMP9 are promoting the growth and development of ESCC. The expression of these associated genes—MMP9, AMPK, and YAP—in the cultivated TE-1 cells under various conditions was examined in order to validate and further analyze the endocrine mechanism resulting from obesity.In the cells cultivated with conditioned media from the obese

group, AMPK mRNA expression dramatically decreased (P < 0:05), while YAP and MMP9 mRNA expression significantly increased (P < 0:05). AMPK protein expressionand p-AMPK significantly decreased (P < 0:05); in the context of obesity, MMP9, totalYAP, and p-YAP protein were significantly increased (P < 0:05) in TE-1 cells (Figure 8).Subsequent investigation also showed that nonphosphorylated YAPexpression continued to increase in TE-1 cells cultivated with conditioned media from the obese group compared to the thin group (20:0 ± 0:9% GAPDH versus 33:4 ± 0:5). Therefore, through an endocrine influence, obesity may enhance the migration, invasion, and proliferation of TE-1 cells as well as change gene expression.

4. Discussion

Ancer risk that can be prevented, like ESCC, is increasingly linked to obesity and increases risk for a number of catastrophic conditions. Given the variety of biological impacts of obesity, including tumor microenvironment, aberrant adipokine production, oxidative stress, metabolites, inflammation, immunology, and complex effects, the pathways relating obesity to ESCC are intricate [12]. These effects fall into the following categories: paracrine, auto-crine, endocrine mechanism, and cancer-stromal interaction [30]. The development of these fat-associated malignancies may be fundamentally influenced by altered metabolicactivity of visceral AT in obesity, andBy promoting cancer cell proliferation or invasion and reducing apoptosis, AT may have an impact on the course of ESCC [31].The growth, invasion, and metastasis of many malignancies, including EC, depend on the cancer-stromal interaction, which is mediated by cell-cell contact and autocrine, paracrine, and endocrine processes [32].Therefore, in order to ascertain the correlation between obesity and ESCC in the study, we built modeling ESCC in vivo and cell trials in vitro using mouse sera. The findings showed that tumors grown from TE-1 xenografts got larger inCompared to lean animals, obese nude mice showed a stronger positive connection with ESCC growth, offering compelling statistical evidence for the direct relationship between obesity and ESCC growth. The findings additionally shown that obesity may enhance TE-1 cell migration, invasion, and proliferation through an endocrine mechanism. The expression of proproliferative and antiapoptotic genes was upregulated in obesity, which helped TE-1 survive.This work used a brand-new in vivo ESCC growth animal model. against the backdrop of obese naked mice. Even though nudemice are not prone to obesity, they are strong candidates for xenograft tumors, which makes them appropriate study subjects.

Based on the criterion, up to 35% (14/40) of the mice were selected as fat mice for additional research. The model may also be utilized to investigate the mechanisms in more detail.of the impact of obesity on ESCC development. Many key mechanisms and metabolites, including insulin, insulin resistance, leptin, and visfatin, are involved in tumorigenesis in obese patients [33, 34]. In this study, there was a strong positive correlation found between the growth of xenograft tumors and serum levels of glucose, HOMA-IR, leptin, and visfatin. In spite of these results, it has to be investigated which specificThe formation and growth of cancer are related to adipose depots or obesity effects.Adipokines, including leptin and adiponectin, have been linked to angiogenesis, cell growth, proliferation, cell cycle regulation, and angiogenesis [12]. The findings of this investigation further corroborated the association between leptin and ESCC growth. Visfatin is one adipokine that has garnered increasing attention recently due to its potential significance in the development of cancer. Visfatin has the potential to induce tumor growth and metastasis in several cancers, including gastric cancer [37], mouth squamous cell carcinoma [36], and breast cancer [35]. This study also demonstrated the connection between visfatin and ESCC development. Additionally, visfatin functions as an enzyme that synthesizes NAD, which was overexpressed incertain malignancies, including as oral squamous cell carcinoma [39] and stomach cancer [38].

Adiposity, hyperinsulinemia, and inflammation are negatively correlated with adiponectin, another well-known adipokine that is primarily released by visceral AT. By activating AMPK, adiponectin may have anticancer effects [40]. AMPK is a crucial sensor of cellular energy that is triggered by elevating AMP levels. It also functions by balancing the availability of energy with the development of cells.deactivated when overweight. Therefore, the AMPK-YAP signaling pathway has a tight relationship with the energy condition of cells, including the metabolism of fats and carbohydrates, which includes obesity and cancer. This work is the first to demonstrate how the AMPK-YAP signaling pathway amplified the development and invasion of ESCC. It also shows that obesity upregulated the expression of MMP9, total YAP, p-YAP, and nonphosphorylated YAP protein while downregulating AMPK and p-AMPK. Additionally, it was discovered that obesity increased the expression of MMP9 and YAP at the mRNA level and decreased AMPK through a complex method that included the endocrine system.Insulin may also affect obesity-mediated cancers including ovarian and kidney tumors [42, 43], and IR was linked to an increased risk of lung cancer [41]. However, the connection between insulin and ESCC growth has not been studied inthe research. The study's groundbreaking discoveries, which showed a positive correlation between blood levels of visfatin and leptin and the formation of xenograft tumors, were in line with the theory that growth factors and adipokines produced in the context of obesity may stimulate the growth and survival of cancer cells. There were undoubtedly some limitations to the study, including the fact that nude mice have a tiny volume of serum that cannot be used for additional analysis, such as lipid and adiponectin assays, and that they are insensitive to creating an obese model. Another is that it is impossible to quantify the complex impact of obesity on the survival of mice harboring malignancies.

5. Conclusion

IR and the altered adipokine milieu linked to obesity may increase the formation of ESCC xenograft tumors and encourage ESCC cell invasion, migration, and proliferation in vitro.

Data Availability

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The raw data of the correlation study between the weight of xenograft tumors and the body weight, liver weight, HOMA-IR, leptin, visfatin, and blood glucose of mice—which was not included in the manuscript—can be obtained upon request from the contributing authors. Every other piece of information is given

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