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Original Article

Association of adenovirus 36 and gynecomastia development: A case-control study

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Abstract

Aim: The interaction of various adipokines secreted from adipose tissue during Adenovirus 36 (Ad-36) infection may cause gynecomastia. In this study, we aimed to evaluate the association between Ad-36 and gynecomastia development by determining DNAs and antibodies of Ad-36, adiponectin, leptin, and IL-6 levels.

Materials and Methods: Patient and control groups were composed of from 33 male adults with gynecomastia and 15 male adults with anatomic disorders without gynecomastia, respectively. Serum neutralization assay (SNA) was used for the determination of Ad-36 antibodies and PCR method was used for the determination of Ad-36 DNA.

Results: No Ad-36 DNA was found in the breast tissue samples. Ad-36 antibody levels were detected in the patient group with gynecomastia higher than the control group ($p<0.05$). The leptin and adiponectin levels were increased and decreased in the Ad-36 positive patient group individuals. IL-6, cholesterol, and triglyceride levels were not detected to be statistically significant between Ad-36-positive and Ad-36-negative patient group individuals ($p>0.05$). Serum leptin and adiponectin levels were detected to be significantly different from each other ($p<0.05$).

Conclusion: In conclusion, a low-grade chronic inflammation may be caused by Ad-36 infections, leading to increased circulating leptin levels. Consequently, leptin may lead to an increase in local or circulating estrogen levels by stimulating the aromatase enzyme in breast tissue and adipose stromal cells.

Keywords: Adenovirus 36; gynecomastia; leptin; serum neutralization assay

INTRODUCTION

Gynecomastia is a common disorder of males, characterized by benign enlargement of breast tissue due to the proliferation of glandular tissue. It is usually a transient condition, but when prolonged it is called gynecomastia. During recent years, the frequency of gynecomastia has increased 3236% in males (1,2). The development of gynecomastia is associated with certain clinical conditions, such as hyperthyroidism, testicular disease, lung cancer, and hepatic cirrhosis. Gynecomastia is also associated with elevated serum progesterone and estrogen concentrations (3,4). In addition, it presents idiopathically in 25% of cases (2). In recent years, androgen resistance due to the development of local adipose tissue and an increase in aromatase enzyme activity are believed to be the causes of gynecomastia (1,4).

Mammary glands cause breast enlargement in the glandular type of gynecomastia. Therefore, the glandular type of gynecomastia must be differentiated from other types of gynecomastia, in which fat deposition increases along with an increase in body mass index (BMI), and increased mammary gland and adipose tissue proliferation (3).

Globally, obesity is an ongoing problem due to a rapid increase in the prevalence of related comorbidities (5). Excessive nutrition and inactivity, genetic, metabolic, hormonal, psychological, socioeconomic, and socio-cultural factors may have a role in the progression of obesity (6). Recently, Adenovirus 36 (Ad-36) antibody titers have been commonly found throughout society and Ad-36 is suggested as a factor in obesity development by causing an increase in adipose tissue. Relationship

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between Ad-36 and obesity was first indicated in animals by showing adipose tissue affinity for Ad-36 (7,8). In human serology studies, this relationship was determined using the serum neutralization assay (SNA) (9-11).

We suggested that Ad-36 may be associated with gynecomastia development, inducing regional adiposity. We also investigated the presence of Ad-36 DNA in breast reduction samples obtained from adult gynecomastia patients using PCR techniques. We investigated Ad-36 antibodies by performing the standard SNA. We also measured serum leptin, adiponectin, and Interleukin 6 (IL-6) levels using ELISA in serum samples taken simultaneously.

MATERIALS and METHODS

Patient group (PG)

Thirty-three adult male patients (mean age = 26.42 ± 8.21) with BMI ≤ 25 kg/m^2 were the patients of a plastic reconstructive and aesthetic surgery clinic. 27 patients were diagnosed with mixed type and 6 patients were diagnosed with glandular type gynecomastia. According to the Simon classification, 18 patients were stage 2A, 12 were stage 2B, and 3 were stage 3 (12).

Control group (CG)

For the control group, we also included 15 adults (mean age = 29.13 ± 11.04) with BMI ≤ 25 kg/m^2, who were admitted to the same hospital during the same period for different reasons, such as anatomic disorders (eg. cleft lip, hexadactyly) without any other organic disease or gynecomastia pathology.

Biochemical tests were used for the serum value measurement of cholesterol, and triglycerides and data were assessed after matching the two groups for age and BMI (p>0.05) (Table 1).

Table 1. Characteristics of patient and patient control groups

	Patient group	Patient control group
Number	33	15
Age	26.42 ± 8.21	29.10 ± 11.04
BMI (kg/m^2), mean ± sd	24.18 ± 1.074	24.20 ± 1.32

BMI; Body mass index, Mean ± sd; mean ± standard deviation

Breast Reduction and Serum Samples

Approximately 160 g of breast reduction sample was obtained from each patient with gynecomastia using lipoaspiration/subcutaneous mastectomy. Adipose tissue samples from the patient control group were obtained subcutaneously. Serum samples of all groups were collected before surgery and tested for the determination of Ad-36 antibody by the SNA. Measurement of leptin, adiponectin, and IL-6 levels were performed by ELISA method. Serum and tissue samples were stored at -80°C.

Assays

1) Immunological assays

a) Serum neutralization test for Ad-36 antibodies

Virus titration: Ad-36 (ATCC-VR-1610) strain as obtained from the American Type Culture Collection (ATCC, Rockville, USA). Ad-36 ATCC-VR-1610 strain was incubated in A549 cell culture. The virus titer was measured by considering the cytopathic effect observed in 50% of the wells including A549 cells. The tissue culture infectious dose of Ad-36 was calculated by serial 10-fold dilutions of Ad-36 ATCC-VR-1610 strain stock solution (13).

Serum neutralization assay: The assay was performed as described previously (7). In this method, test sera were inactivated by heat at 56ºC for one hour then 1:4 to 1:512 serial dilutions were performed. Afterwards, the virus suspension was equilibrated to 37°C. Later, Ad-36 stock solution was mixed with serum samples in a ratio of one-to-one and incubated for one hour at 37ºC. One hundred µl of this mixture was then added to each well containing A549 cells ($2x10^4$ cells per well). All sera were run in triplicates. Ad-36 including cells without serum and only cells including samples were inoculated to each plate. Incubated plates were allowed to stay at 37°C in CO_2 (5%) for 10 days. The developed CPEs were evaluated in dilutions by comparing the CPE in control wells. Sera with no CPEs in dilutions were regarded as positive (7).

b) Measurement of leptin, adiponectin and IL-6 levels in sera

Measurement of leptin, adiponectin, and IL-6 levels were performed by using a commercial ELISA kit.

2) PCR

Approximately 1 g of tissue sample was ruptured using a sterile scalpel. Ad-36 DNAs were obtained by using a DNA extraction kit (Roche Diagnostics GmbH, Germany). The resulting extractions were stored at -80°C until further use. Ad-36 DNA was monitored by in-house PCR (single step method) and nested PCR by primers designed for Ad-36 *E4 orf1* gene as described previously (8,14).

In PCR assays, the reaction volume was 50 µl, which contained 10 µl DNA extract. A PTC-100 thermal cycler (MJ Research Inc, USA) was used. PCR reactions were as follows; one cycle for 2 minutes (95°C); 35 cycles for one minute (94°C), one minute (58°C), and for two minutes (72°C) and one cycle for five minutes (72°C). The expected size of PCR products was ~138 bp for in-house PCR and ~650 bp for nested-PCR. ATCC-VR-1610 (Ad-36) was positive control.

Ethics

This study was approved by the Non-Invasive Clinical Research Ethics Committee of Bagcilar Research and Training Hospital (Number: 2012-27). All patients provided their informed consent to participate in the study.

Statistical analysis

Comparison of the frequencies and percentages between the study groups were analyzed by Fischer's exact test. Comparisons were done by Mann-Whitney U test.

In addition, a logistic regression test was applied for multivariate analysis. IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp. Armonk, NY: USA. Released 2012) was used for statistical analyses and the values were accepted as statistically significant when p<0.05.

RESULTS

A statistically significant difference was detected between the patient and control groups for cholesterol levels, but no statistically significant difference was detected for triglyceride, leptin, adiponectin, and IL-6 levels (Table 2).

Table 2. Test results for patient and patient control group

	Patient group	Patient control group
Serum cholesterol (mmol/L)	174.8 ± 21.74	144.9 ± 36.86*
Serum triglyceride (mmol/L)	115.7 ± 54.6	123.3 ± 45.78**
Leptin (ng/ml)	16.50 ± 19.8	6.31 ± 3.2**
Adiponectin (µg/ml)	22.75 ± 16.00	22.73 ± 8.7**
IL-6 (pg/mL)	2.415 ± 1.33	1.29 ± 0.37**
Ad-36 Ab (positive/negative)	8/33	0/15*

Ad-36 Ab: Adenovirus-36 Antibody; Mean ± sd; *p < 0.05; **p > 0.05

Ad-36 antibody was found in 8 of 33 (24.2%) serum samples of the patient group by SNA. In the CG, Ad-36 antibodies were not detected. A significant difference was observed for the existence of Ad-36 antibody between the patient and the control groups (p<0.05) (Table 2).

Table 3. Comparison of the leptin, adiponectin, IL-6, serum cholesterol and triglyceride levels in patients with gynecomastia based on Ad-36 antibody presence

	The presence of Ad-36 Ab		Statistical value
	Ad-36 Ab (+) (n=8)	Ad-36 Ab (-) (n=25)	
Leptin (n:33)	48.94 ± 12.63	6.124 ± 3.74	p <0.0001
Adiponectin (n:33)	3.98 ± 1.85	28.76 ± 13.61	p <0.0001
Serum cholesterol (n:33)	170.4 ± 17.31	176.3 ± 23.11	p >0.05
Serum triglyceride (n:33)	135.1 ± 57.22	109.4 ± 53.41	p >0.05
IL-6 (pg/mL)	1.10 ± 3.105	4.62 ± 6.75	p >0.05

There was a significant difference (p<0.0001) for leptin and adiponectin levels between the Ad-36 antibody positive and antibody negative samples of the patient group. Mean leptin and adiponectin levels were increased and decreased in the serum samples of the patient group individuals, respectively. Although serum cholesterol levels were detected in lower concentrations in Ad-36 antibody-positive patients (p>0.05), it was not considered as statistically significant. According to the data we obtained, there were no significant differences for IL-6 levels between Ad-36 antibody positive and Ad-36 antibody negative patient group individuals (Table 3).

In the logistic regression analysis with demographic properties, biochemical parameters, and Ad-36 antibody positivity, only the serum cholesterol level was determined to be a risk factor for gynecomastia (p<0.004). However, a significant difference was detected using univariate analysis for Ad-36 antibody positivity and has been identified as a risk factor (Table 4).

Table 4. Logistic regression of variables

	Variables in the Equation					
	B[a]	S.E.[b]	Wald	df[c]	Sig.[d]	Exp (B)
Ad-36	1.123	2.047	0.301	1	0.583	3.073
Age	0.057	0.049	1.361	1	0.243	1.059
Triglyceride	0.011	0.010	1.394	1	0.238	1.011
Cholesterol	-0.050	0.017	8.120	1	0.004	0.952
Adiponectin	-0.034	0.039	0.758	1	0.384	0.966
Leptin	-0.128	0.074	3.004	1	0.083	0.880
Constant	6.750	3.281	4.234	1	0.040	854.315

[a] B; beta regression coefficient, [b] S.E.; standard error; [c] df; degree of freedom, [d] Sig; significance

No DNA of Ad-36 was found in the tissue samples of the all study groups. No inhibitor was detected in tissue samples.

DISCUSSION

HIV, HCV, and MMTV viruses have been thought to be associated with the development of gynecomastia (15-17). Elevated estrogen and reduced testosterone levels caused by hypogonadism and drug therapy for HIV infections have been suggested as the cause of gynecomastia. On the other hand, Biglia et al., (17) suggested that hypogonadism was the major cause of gynecomastia development in HIV infected rather patients than adverse effects of antiretroviral drugs (18). Moreover, testosterone level reductions as a consequence of liver cirrhosis in patients with HCV infection may cause the development of gynecomastia (15). Ford et al., (16) indicated significant mouse mammary tumor virus-like gene sequences in breast tissues of male patients with gynecomastia and concluded that mouse mammary tumor virus positivity may be related to hormonal imbalances in breast tissues of males with gynecomastia. Currently, this mechanism for the gynecomastia development is suggested as increased endogenous estrogen with increased androgen inhibition, leading to an increase in the proliferation of breast tissue (19-21).

Androgens are primarily aromatized to estrogens in breast adipose tissue during breast development. It may cause in

situ tissue proliferation because the aromatase enzyme of the breast adipose tissue may contribute to gynecomastia development (3). We hypothesized that by changing the hormone balance from androgens to estrogens, Ad-36 may cause the development of gynecomastia in males. In other words, the mechanism related to the gynecomastia development may be similar with the mechanisms which are hypothesized for the association of Ad-36 and obesity development. We met only one other study in the literature survey supporting our hypothesis, published in Medical Hypothesis (IL-6 was added to this study) (22). Therefore, in the evaluation of our data, we compared our results with other Ad-36-obesity-related studies.

In the hypothesized Ad-36-obesity association, the interactions between the immune system and adipose tissue were suggested as the cause of enlargement of fat tissue. It consequently leads to inflammation by altered immune response. In addition, alterations in adipokine secretion may have been seen during these interactions (23,24). Primary human adipocytes of stem and stromal cells may be infected by Ad-36 (25). Ad-36 was suggested to have tropism for adipocyte cells by fiber proteins. Ad-36's E4 (ORF1) viral gene expression initiates this mechanism by facilitating the entrance of Ad-36 to the nuclei of host cells. Consequently, the promotion of adipogenesis may be seen by the acceleration of adipocyte proliferation and differentiation (26-28). Obesity development has been regarded as a chronic inflammatory process (24-29). In most of these studies, the existence of the Ad-36 antibody was determined by using the SNA as the optimal diagnostic test (accepted as gold standard) with high sensitivity and specificity, often used in serologic-based studies of obese adults (9,11).

In our study, according to univariate analysis, we observed a significant difference ($p<0.05$) for Ad-36 antibody levels between the patient the patient control groups. No Ad-36 DNA was detected in the breast tissue samples of patients. On the other hand, mean leptin and adiponectin levels were detected as significantly higher and lower in Ad-36 antibody positive patients according to the univariate analysis, respectively. On the other hand, it was shown that adipocytes infected with Ad-36 secrete various cytokines, like IL-6 and TNF-α, in obese individuals. Therefore, we aimed to evaluate the presence of IL-6 together with leptin and adiponectin. Berger et al., (30) reported high IL-6 levels in obese children, but Karamese et al., (31) reported higher IL-6 levels both in obese adults and children, but they did not detect any significant difference between their groups. We also found higher IL-6 levels in Ad-36 positive patients than Ad-36 negative patients, similar to the results of Karamese et al., (31) study.

There was a significant difference for the leptin levels between Ad-36 antibody-positive patient group individuals (48.94 ng/ml) compared to the Ad-36 antibody-negative patient group individuals (6.124 ng/ml). IL-6 may be increased in adipocytes as a result of Ad-36 infections and may be the cause of a low-grade chronic inflammation (32). Macrophage infiltration into the adipose tissues may be stimulated by monocyte chemoattractant protein-1 (MCP-1) and followed by the production of proinflammatory factors from the adipose tissues (33-35). Hypertrophic adipocytes may also have the capability to produce plasminogen activator inhibitor-1, MCP-1, TNF-α, and resistin (19-20). Pro-inflammatory cytokines may increase leptin levels, and the low-grade chronic inflammation that exists after the disappearance of Ad-36 (21). Therefore, we may hypothesize that local or circulating estrogens can be increased by leptin and leptin can also shift the androgen/estrogen balance on behalf of estrogen by activating aromatase enzyme activity in breast tissue and adipose stromal cells. A different hypothesis is that leptin may directly affect the mammary epithelial cells by stimulating growth. Alternatively, the estrogen signal of breast tissue may be amplified by estrogen receptor activation via leptin (36). We may suggest that Ad-36 may be implicated in the regional adipose tissue increase similar to the mechanism as hypothesized in the obesity-Ad-36 association. In a plausible hypothesis, higher aromatization of androgens to estrogens by the endocrine function of peripheral adipose tissue may cause the progression of the male gynecomastia (37). Adipose tissue may produce leptin and estrogen hormones as an endocrine organ (38,39). Production of excessive estrogens in the male breast tissue may lead to male gynecomastia (40).

CONCLUSION

In conclusion, a low-grade chronic inflammation may be caused by Ad-36 infections, leading to increased circulating leptin levels. Consequently, leptin may lead to an increase in local or circulating estrogen levels by stimulating the aromatase enzyme in breast tissue and adipose stromal cells. Additional comprehensive serial, matched-based, case-control and cohort-based studies with multiple patients are needed to further investigate the association between Ad-36 and gynecomastia development.

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Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study was approved by the Non-Invasive Clinical Research Ethics Committee of Bagcilar Research and Training Hospital (Number: 2012-27).

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