Laughter up-regulates the genes related to NK cell activity in diabetes

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ABSTRACT

To elucidate the sustainable effects of laughter on gene expression, we recruited type 2 diabetic patients who were in-patient for receiving self-management education and examined time-dependent regulation for gene expression by laughter. Two-day experiment was performed. On one day, the patients watched comic video and laughed together with hospital staffs. On the other day, they participated in an inpatient diabetes educational program. Blood samples were collected before and 1.5, 4 h after watching comic video or spending lecture time, and changes in gene expression were comprehensively analyzed by microarray technique. Of the 41,000 genes analyzed, the laughter relatively up-regulated 39 genes, among which, 27 genes were relatively increased in the expression for all the observation period after watching comic video. By functional classification of these genes, 14 genes were found to be related to natural killer cell activity. No genes were included that are directly involved in blood glucose regulation, though successive suppression of postprandial blood glucose levels was observed. These results suggest that the laughter influences the expression of many genes classified into immune responses, and may contribute to amelioration of postprandial blood glucose elevation through a modulation of NK cell activity caused by up-regulation of relating genes.

There are several studies on the physiological effects of laughter that have mainly focused on immunological aspects (1, 2, 22, 24) since Cousins, who had overcome his own disease, first reported that laughter was beneficial to the human body (5). On the other hand, negative emotions are well known to elevate blood glucose levels. With a hypothesis that laughter, which is one of the indicators for positive emotions, potentiate to suppress increases in blood glucose levels, we conducted a study on patients with type 2 diabetes, and the results showed that

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laughter markedly suppressed the increase in the postprandial blood glucose (PPBG) levels (10) and influenced the gene expression profile in the peripheral blood leukocytes (11). Furthermore, our consecutive study suggested that laughter prevents the exacerbation of diabetic nephropathy (12).

In the present study, we analyzed the time-dependent changes in gene expression by laughter using a microarray technique, to identify responsible genes

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for the beneficial effect of laughter on blood glucose regulation in the patients with type 2 diabetes.

MATERIALS AND METHODS

Subjects. Ten type 2 diabetic patients who were in-patient for receiving self-management education and skill training (6 men and 4 women) with a mean (\pm SD) age of 55.3 \pm 10.0 years, body mass index of 24.1 \pm 4.2 kg m⁻², and HbA_{1c} of 7.7 \pm 0.9% participated in a 2-day experiment. The experimental protocol was approved by the medical ethics committee of Tenri Yorozu-sodansho Hospital (Nara, Japan). Before participation, the purpose and risks associated with this study were carefully explained to all of the subjects, and written informed consents were obtained.

Study design. All patients underwent blood collection to determine basal levels of blood glucose and gene expression before lunch, and took the same meal on consecutive days. Patient meals were individually adjusted in energy according to their desirable body weight and in the same proportion of carbohydrate, protein, fat and fiber to the total energy. After a meal on day 1 or day 2 of the experiment, the patients watched a comic show video which featured well-known Japanese comedians and laughed together with other patients and hospital staffs for 1 h. On the other experiment day, they spent one tedious diabetes lecture hour after lunch devoid of laughing episodes (control). The subjects were randomly assigned to watch comic video either on the first day or the second day. To measure blood glucose, blood samples were collected immediately, 1.5 and 4 h after watching comic video or spending lecture time (corresponding to 2, 3.5 and 6 h after the start of the meal). To analyze gene expression, blood samples were obtained 1.5 and 4 h after watching comic video or spending lecture time.

Microarray hybridization and data acquisition. RNA samples from the five subjects who showed successive suppression of the increase in PPBG were mixed and then comprehensively analyzed by microarray technique. Total RNA was prepared from the blood samples using PAXgene Blood RNA Kit (Qiagen) and reverse-transcribed to cDNA containing a T7 RNA polymerase promoter sequence. Complementary RNA (cRNA) incorporated aminoallyl nucleotides were generated from the cDNA by *in vitro* transcription and labeled with cyanine (Cy) 3 or Cy5 dyes (14, 16). Cy3-labeled cRNA from samples

before watching comic video or spending lecture time was mixed with an equal amount of Cy5labeled cRNA from samples taken 1.5 or 4 h after watching comic video or spending lecture time for the analysis of the genes exhibiting relative changes in expression. The mixture was applied to the microarray spotted 41,000 genes (including transcripts) (Agilent Whole Human Genome), and hybridization was allowed to proceed for 17 h at 65°C as described by the manufacturer. After hybridization, the arrays were washed, and scanned using a confocal laser scanner (Agilent G2565BA). The fluorescence intensities on the scanned images were quantified, corrected for background, and normalized using global normalization methods based on the assumption that the median values of the fluorescence intensities of both samples were identical. Array data were deposited at the Gene Expression Omnibus (National Center for Biotechnology Information) with Accession No. GSE4901.

For the analysis of the genes exhibiting relative changes in expression, genes were selected which displayed differences of more than 1.25- and 1.5-fold, or 0.8- and 0.67-fold expression changes before and 1.5 h and 4 h after watching the comic video and those before and after spending lecture time, respectively.

RESULTS

The increase in the 2-h PPBG after watching the comic video was suppressed in 7 patients (mean: $37.4 \pm 23.9 \text{ mg dl}^{-1}$, P = 0.028), but not in 3 patients. Successive suppression of PPBG for 4 h after watching the comic video was observed in 5 patients.

Of the 41,000 genes analyzed, the exposure to comic video induced relative changes in the expression of 40 genes. Thirty nine genes were relatively up-regulated and one was down-regulated. Among 39 up-regulated genes, 27 were relatively increased in the expression for all the observation period after watching comic video.

By functional classification of these genes exhibiting continuous increase in the expression, immune-related genes (15 genes) were most frequent (Table 1). Next in frequency were signal-transduction-related genes (7 genes). There were no genes included that should be directly involved in blood glucose regulation. Fourteen out of 15 immune-related genes are supposed to be connected with natural killer (NK) cell activity.

No. Gare Symbol Gare bymol Gare bymol Gare Symbol Gare Symbol Hs 1051 1144 18 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371 1371					Relative char	nge (-fold)	
1 GZMB gramzyme B Hs. 1051 144 198 In 2 CST7 cystatin F Hs. 143212 127 157 In 3 EDG8 endohelial differentiation, sphingolipid G-protein-coupled receptor, 8 Hs. 143212 127 155 Mi 6 KIRZDIA killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 105 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 106 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 107 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 107 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 107 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 133 107 Immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 312227 133 118 153 153 153 153 153 153 153 153 153 153 153 153 153 153 1	No.	Gene Symbol	Gene Name	Unigene No. —	1.5 h	4 h	Functional category
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	-	GZMB	granzyme B	Hs. 1051	1.44	1.98	Immune response
3 EDG8 endothelial differentiation, sphingolipid G-protein-coupled receptor, 8 Hs. 501561 1.43 1.85 Sig 6 KIR3D2 eirbolytate (N-acetylgucosanine-G-O) sulforansferase 2 Hs. 8786 1.27 1.55 Mm 7 KIR3D12 killer cell immungolobulin-like receptor, three domains, short cytoplasmic tail, 2 Hs. 53437 1.31 1.06 hm 7 KIR3D12 killer cell immungolobulin-like receptor, three domains, long cytoplasmic tail, 2 Hs. 53437 1.31 1.06 hm 8 GN1X graular of G-protein signalling 9 Hs. 1005806 1.33 1.171 hm 11 KIR3D12 killer cell immungolobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 51237 1.30 1.23 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.33 1.34 1.33 1.33 1.36 1.33 1.34	7	CST7	cystatin F	Hs. 143212	1.27	1.57	Immune response
4 CHST2 carbohydrate (N-acetylglucosamine-6-O) sulfortansferase 2 Hs R8786 1.27 1.55 MM 5 KR2DD2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 53437 1.31 1.68 Imm 7 KR3DD2 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 53437 1.31 1.68 Imm 9 GNIX granulysin Hs 74050 1.33 1.71 Imm 9 GNIX granulysin Hs. 123277 1.30 1.52 Si Immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 132327 1.33 1.53 Imm Immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 148505 1.93 Immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 132327 1.33 1.53 Immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 312327 1.33 1.53 Immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 31087 Hs. 3108	б	EDG8	endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	Hs. 501561	1.43	1.85	Signal transduction
5 KIR2DS4 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 4 His. 258612 1.38 1.97 hm 7 KIR2D12 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 His. 354343 1.31 1.06 hm 7 KIR3D12 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 His. 363463 1.45 1.95 hm 9 RG39 regulator of G-protein signalling 9 His. 105806 1.33 1.71 hm 10 KIRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 His. 123277 1.30 1.52 Si 11 KIRZDS killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 His. 12078 1.43 1.83 hm 13 KIRZDS killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 His. 12078 1.43 1.78 1.68 56 14 72R coagulator factor D His. 492562 1.42 1.58 1.67 56 1.78 56 1.68 56 <t< td=""><td>4</td><td>CHST2</td><td>carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2</td><td>Hs. 8786</td><td>1.27</td><td>1.55</td><td>Metabolism</td></t<>	4	CHST2	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	Hs. 8786	1.27	1.55	Metabolism
6 KR2DL2 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 Hs. 334327 1.31 1.68 Im 7 KR3DL2 killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2 Hs. 34563 1.45 1.95 Im 9 RGS9 regulator of G-protein signalling 9 Hs. 105806 1.33 1.71 Im 1 KIRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 74082 1.33 1.53 Im 1 KIRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 74082 1.38 1.53 Im 1 KIRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 74082 1.38 1.53 Im 1 KIRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 74082 1.38 1.53 Im 13 KIRC3DS1 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 74082 1.30 1.53 Im 14 KIR2DS2 G provin/r	5	KIR2DS4	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 4	Hs. 258612	1.38	1.97	Immune response
7 KIR3DL2 killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2 Hs. 54263 1.45 1.95 hm 8 GNLY granulysin Hs. 105806 1.33 1.71 hm 9 KIR2DS killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 13227 1.33 1.53 hm 11 KIR2DS1 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 512574 1.38 1.83 hm 13 KIR2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 512574 1.38 Km 1.83 1.36 Hs. 1.83 1.36 Hm 1.30 1.78 Si Hm 1.30 1.78 Mm 1.30 1.78 Mm 1.83 5.53 Hm 1.83 5.53 Hm 1.83 5.53 Hm 1.73 1.84 1.30 1.77 Mm 1.84 1.83 5.53 Hm 1.55 Hm 1.55 Hm 1.55 Hm 1.55	9	KIR2DL2	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2	Hs. 534327	1.31	1.68	Immune response
8 GNLY granulysin Hs. 105806 1.33 1.71 Im 9 RGS9 regulator of G-protein signalling 9 Hs. 13227 1.30 1.52 Si 10 KLRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 13227 1.30 1.52 Si 11 KR2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 7402 1.33 1.83 Im 13 KR2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 74134 1.30 1.58 Mm 13 KR2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 74134 1.30 1.58 Mm 14 F2R coagulation factor II Hs. 482562 1.42 1.58 Si 16 PDGFD platelet derived growth factor D Hs. 513633 1.30 1.62 Gr 17 RR1 natual cytotoxicity triggering receptor 1 Hs. 42562 1.42 1.58 Si 18 NCR1 <td>7</td> <td>KIR3DL2</td> <td>killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2</td> <td>Hs. 546263</td> <td>1.45</td> <td>1.95</td> <td>Immune response</td>	7	KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2	Hs. 546263	1.45	1.95	Immune response
9 RGS9 regulator of G-protein signalling 9 Hs. 13227 1.30 1.52 Si 10 K1RC3 killer cell immunoglobulin-like receptor, beta polypeptide Hs. 74082 1.33 1.53 Im 11 K1R2DS1 killer cell immunoglobulin-like receptor, beta polypeptide Hs. 51274 1.38 1.83 1.83 Im 12 PKBRDS killer cell immunoglobulin-like receptor, beta polypeptide Hs. 51374 1.30 1.78 Si 13 R1R2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 513633 1.40 1.78 Si 14 F2R coagulation factor II receptor Hs. 513633 1.36 Hs. 513633 1.30 1.78 Si 16 PDGFD platelet derived growth factor D Hs. 513633 1.36 1.80 Si Im Hs. 513633 1.30 1.77 Im 17 PRF1 perforin natural cytoxicity triggering receptor 1 Hs. 4176484 1.27 1.55 Im 18 OCFI Aproto	∞	GNLY	granulysin	Hs. 105806	1.33	1.71	Immune response
10 KLRC3 killer cell lectin-like receptor subfamily C, member 3 Hs. 74082 1.33 1.53 Im 11 KRRC3 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 1 Hs. 712574 1.38 1.83 hin 12 PDGFRB platelet-derived growth factor receptor, two domains, short cytoplasmic tail, 2 Hs. 7134 1.30 1.78 Sit 13 KIR2DS2 killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2 Hs. 74134 1.30 1.78 Sit 14 FPR6 coagulation factor II receptor Hs. 513633 1.30 1.78 Sit 17 PR1 perforin 1 Hs. 23038 1.30 1.62 Or 17 PR1 perforin 1 Hs. 2700 1.99 1.79 Im 17 PR1 perforin 1 Hs. 416848 1.30 1.62 Or 18 NCR1 natural cytotoxicity triggering receptor 1 Hs. 47084 1.27 1.55 Im 18 NCR1 natural cytotoxicity triggering receeptor	6	RGS9	regulator of G-protein signalling 9	Hs. 132327	1.30	1.52	Signal transduction
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21 KSP37 Ksp37 protein 22 KSP1B SH2 domain containing IB 22 SH2DIB SH2 domain containing IB 23 CCL4 chemokine (C-C motif) ligand 4 24 GZMH granzyme H 25 TBX21 T-28 1.52 26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.55 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sig	20	SPON2	spondin 2	Hs. 302963	1.31	1.84	Cell adhesion
22 SH2D1B SH2 domain containing 1B Hs. 350581 1.35 1.62 Sig 23 CCL4 chemokine (C-C motif) ligand 4 Hs. 75703 1.28 1.52 Im 24 GZMH granzyme H Hs. 348264 1.35 1.54 Im 25 TBX21 T-box 21 Hs. 272409 1.26 1.52 Tr 26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.56 - 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.56 -	21	KSP37	Ksp37 protein	Hs. 98785	1.33	1.77	Immune response
23 CCL4 chemokine (C-C motif) ligand 4 Hs. 75703 1.28 1.52 Im 24 GZMH granzyme H Hs. 348264 1.35 1.54 Im 25 TBX21 T-box 21 Hs. 272409 1.26 1.52 Tr 26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.56 - 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sig	22	SH2D1B	SH2 domain containing 1B	Hs. 350581	1.35	1.62	Signal transduction
24 GZMH granzyme H 1.54 Im 25 TBX21 T-box 21 1.54 1.54 Im 26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.52 Tr 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sit	23	CCL4	chemokine (C-C motif) ligand 4	Hs. 75703	1.28	1.52	Immune response
25 TBX21 T-box 21 Hs. 272409 1.26 1.52 Tr 26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.56 - 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sig	24	GZMH	granzyme H	Hs. 348264	1.35	1.54	Immune response
26 - hypothetical protein FLJ20699 Hs. 435832 1.26 1.56 - 27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sig	25	TBX21	T-box 21	Hs. 272409	1.26	1.52	Transcription factor
27 CLIC3 chloride intracellular channel 3 Hs. 64746 1.26 1.54 Sig	26		hypothetical protein FLJ20699	Hs. 435832	1.26	1.56	
	27	CLIC3	chloride intracellular channel 3	Hs. 64746	1.26	1.54	Signal transduction

Table 1 Functional classification of genes exhibiting continuous increase in expression

Relative change column indicates change ratio 1.5 h and 4 h after watching comic video. NK cell activity-related genes are hatching.

DISCUSSION

While genes that are directly involved in a blood glucose regulation were not discriminated in this study, the laughter up-regulated the many genes for immuno-functional molecules related to NK cell activity. The GZMB and GZMH genes encode granzymes B and H, respectively, which are serine proteases involved in NK cell- or cytotoxic T lymphocyte (CTL)-mediated target cell lysis, and are present in cytoplasmic granules of these cells (13, 15, 18, 20). Perforin encoded by PRFI is a C9-like protein which can form lesions in the membranes of target cells, and is contained in the cytoplasmic granules of NK cells and CTLs (13, 17, 26). This protein polymerizes in the presence of Ca²⁺ ions to cause rapid depolarization of the target cell membrane as a tubular complex forming large transmembrane pores (23). Granulysin encoded by GNLY is also presented in cytotoxic granules of NK cells and CTLs, and exert anti-microbial and anti-tumor activity (21). Cathepsin W encoded by CTSW is a novel cysteine protease presented in the endoplasmic reticulum of NK cells (25). KLRC3, NCR1 and KIR family (KIR2DS1, S2, S4, L2, KIR3DL2) genes encode killer cell lectin-like receptor, natural cytotoxicity triggering receptor and killer cell immunoglobulin-like receptors, respectively, which should play in regulating NK cell functions (4, 8, 22). KSP 37 encodes killer-specific secretory protein at late pregnancy, and may play important roles near parturition (9). CCL4 encodes a chemokine produced by neonatal NK cells in innate immune system (3).

Laughter is well known to enhance NK cell activity (1, 2). Since a comprehensive analysis was performed in this study to discriminate the genes regulated by laughter, NK cell activity was not measured. Recent studies have reported that adoptive transfer of NK cells into ob/ob mice resulted in a significant improvement in glucose intolerance (7) and an improvement of glucose intolerance by oral immune regulation towards liver extracted proteins was associated with elevated intrahepatic NK cells (6). Given that hepatic steatosis was also improved in the report, intrahepatic conditions in good shape, that is, keeping the good environment where constituent cells in the liver operate collaboratively in its functions of immuno-surveillance and nutrient metabolism, are important to adequate postprandial blood glucose regulation. This is consistent with the concept that intra-abdominal fat is responsible for insulin resistance, resulting in the metabolic syndrome that includes glucose intolerance. These

findings lead the present data to the stringent speculations about the mechanism for the potential effect of laughter on blood glucose regulation. Laughter may contribute to amelioration of glucose intolerance through a modulation of NK cell activity caused by up-regulation of relating genes.

On the other hand, the expressions of genes which were functionally categorized in signal transduction were also continuously increased by the laughter. Four out of 7 genes in this category encoded the receptors, suggesting that the laughter may facilitate the communications between functional molecules in the body. However, the exact relationships between laughter and expression changes in some genes exhibiting relative change are still unclear.

Our previous study showed the genes involved in immune response and signal transduction were relatively decreased in their expression by laughter (11). The discrepancy against the relative increase found in the present study may be due to the differences in participants' demography, control conditions or experimental environment between these two studies. The control condition in the previous study was participating in a monotonous academic lecture instead of an inpatient diabetes educational program in this study. In addition, the place where the experiments were undertaken in the previous study was the city hall instead of the meeting room in the hospital in this study.

It is also possible that laughter exerts effects on blood glucose regulation by still unknown routes. Further studies are necessary for identifying the responsible genes for laughter-induced psychosomatic alterations like changes in blood glucose levels.

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