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Elevated Levels of an Angiogenic Factor, Midkine, in Serum of Patients with Hernia

Podwyższone stężenie czynnika naczyniopochodnego, midkiny, w surowicy krwi pacjentów z przepukliną

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Abstract

Background. Gastrointestinal malignancies are among the leading causes of cancer-related deaths in the western world, which is a result of too late detection of on-going carcinogenesis. At the same time, angiogenesis is an essential event for tumour progressive growth and metastasis. Midkine (MK), together with other angiogenic factors, is engaged in this process. During last years its expression in malignancies of gastrointestinal tract and possible application as a tumor marker have been intensively studied, both at m-RNA and protein levels. Hernia patients often serve as controls in studies on disorders of gastrointestinal tract as rapture usually does not cause any changes in most of biochemical parameters. In order to determine if it is also true in case of midkine, the authors studied serum midkine levels in this group of patients in comparison with apparently healthy blood donors.

Objectives. Establishing the serum level of midkine in hernia patients, comparison with healthy subjects and elucidation whether sera from hernia patients can serve as controls in midkine assays.

Material and Methods. The authors measured serum levels of midkine in hernia patients (n = 23) in comparison with apparently healthy subjects (n = 43). In the studies the authors applied a double antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) with antibodies against human midkine from two hosts: rabbit as capture and goat as detection antibodies. Biotin-streptavidin conjugated with horseradish peroxidase amplification-detection system was utilized. Oxidation product of tetramethylbenzidine was measured at 450 nm and the intensity of the developed colour was proportional to midkine concentration in the sample.

Results. The median midkine level in sera from hernia patients was significantly higher compared to healthy blood donors (0.38 ng/ml and 0.14 ng/ml, respectively). In over 43% of studied hernia patients, serum midkine levels were elevated above upper normal range established as 82nd percentile of midkine level in healthy subjects.

Conclusions. In the case of midkine determination, hernia patients should be excluded from control groups as midkine level is already elevated in some patients in this condition (*Adv Clin Exp Med* 2006, 15, 2, 265–269).

Key words: midkine, ELISA, tumour marker, growth factor, hernia.

Streszczenie

Wprowadzenie. Nowotwory przewodu pokarmowego są jedną z głównych przyczyn zgonów związanych z chorobą nowotworową, co w dużej mierze wynika ze zbyt późnego ich rozpoznawania, najczęściej w stadium zaawansowania choroby wykluczającym leczniczą resekcję. Kluczowym procesem w karcynogenezie jest angiogeneza, w którą są zaangażowane czynniki angiogenne, m.in. midkina (MK). W ciągu ostatnich lat intensywnie bada się ekspresję midkiny w nowotworach przewodu pokarmowego zarówno na poziomie białka, jak i mRNA oraz możliwość wykorzystania midkiny jako biomarkera choroby nowotworowej. W wielu doświadczeniach dotyczących chorób przewodu pokarmowego pacjentów z przepuklinami, bez widocznych objawów współistnienia innych chorób, wybiera się jako grupę kontrolną, gdyż uważa się, że obecność przepukliny nie wpływa na większość wskaźników biochemicznych krwi. W celu określenia, czy takie przekonanie jest zasadne odnośnie do czynnika wzrostu midkiny, porównano jej stężenie w surowicy pacjentów z przepuklinami oraz u osób uznanych za zdrowe. **Cel pracy.** Ustalenie stężenia midkiny w surowicy pacjentów z przepuklinami porównanie go ze stężeniem uzyskanym dla grupy osób uznanych za zdrowe i określenie przydatności pacjentów z przepuklinami jako grupy kontrolnej w badaniach nad midkiną.

Materiał i metody. Zmierzono stężenie midkiny w surowicy krwi pacjentów z przepuklinami ($n = 23$) i porównano ze stężeniem midkiny w surowicy osób zdrowych ($n = 43$). Do oznaczeń wykorzystano test immunoenzymatyczny ELISA typu *sandwich* z dwoma rodzajami przeciwciał skierowanych przeciwko midkinie (DASI-ELISA): przeciwciał króliczych jako przeciwciał opłaszczających i przeciwciał kozich jako przeciwciał wykrywających. W teście zastosowano system detekcji i amplifikacji sygnału biotyna–streptawidyna skoniugowana z peroksydazą z chrzanu i tetrametylobenzydynę jako substrat. Produkt reakcji oznaczano kolorymetrycznie przy 450 nm.

Wyniki. W grupie pacjentów z przepuklinami zaobserwowano znacząco większą medianę stężenia midkiny niż w grupie osób uznanych za zdrowe (odpowiednio: 0,38 ng/ml i 0,14 ng/ml). Stwierdzono, że w ponad 43% przypadków pacjentów z przepuklinami, stężenie midkiny w surowicy krwi przekracza górną granicę zakresu normalnego, określonego jako średnia + SD.

Wnioski. Pacjenci z przepuklinami, przynajmniej w badaniach nad czynnikiem wzrostu – midkiną, nie mogą być grupą kontrolną, gdyż obecności przepukliny towarzyszy podwyższone stężenie midkiny w surowicy (*Adv Clin Exp Med* 2006, 15, 2, 265–269).

Słowa kluczowe: midkina, ELISA, marker nowotworowy, czynnik wzrostu, przepuklina.

Midkine is a secreted angiogenic growth factor involved in carcinogenesis and tumor progression [1, 2]. Its engagement in malignancies of gastrointestinal tract has been intensively studied over past few years. Overexpression of this growth factor has been confirmed by immunohistochemical search at the protein level and by RT-PCR as well as in situ hybridisation at the level of nucleic acids in tumours of all parts of gastrointestinal tract [3]. Midkine expression has been found increased as compared to adjacent normal tissue in 80% of esophageal, gastric, hepatocellular and colorectal cancers [2]. Recently, it has also been observed that midkine is released into the bloodstream by cancer cells. An increased level of serum midkine has been associated with number of gastrointestinal cancers [4–6]. In studies on neoplasms of gastrointestinal tract, hernia patients often serve as non-malignant controls, as most of biochemical parameters usually remains unchanged in this condition [7]. However, preliminary studies suggested that midkine levels in hernia patients might be already elevated above the reference range reported in the literature [5, 6]. Therefore, the authors designed this work to establish the serum level of midkine in hernia, compare it to healthy subjects and elucidate whether sera from hernia patients can serve as controls in midkine assays.

Material and Methods

Serum Samples

A group of 23 hernia patients, healthy in other respects, treated between 2004 and 2005 in the Department of Gastrointestinal and General Surgery of Silesian Piasts University of Medicine in Wrocław, was included in the present studies. Blood samples, before surgery, were collected in Sarstedt s-Monovette tubes, allowed to clot at room temperature and were centrifuged at 3,000 rpm for 15 minutes. The resulting sera were frozen

and stored at -25°C until examination. Sera of apparently healthy blood donors ($n = 43$) were kindly provided by Regional Centre of Blood Donation and Therapeutics in Wrocław, Poland and kept frozen (-25°C) until examination. Prior to assay, the serum samples were brought to room temperature and mixed completely. The authors used serum ten times diluted with 0.05% Tween-20 in PBS. Local Medical Ethics Committee approved the project presented in this paper.

Enzyme-Linked Immunoassay

A new DASI-ELISA method for determination of human midkine concentrations in serum was applied [8]. Nunc MaxiSorp microtiter plates were coated with anti-human midkine polyclonal antibodies from rabbit (Gentaur, Belgium) at concentration of 4 $\mu\text{g}/\text{ml}$ in 50 mM carbonate buffer, pH 9.6 and incubated overnight at room temperature on microplate rotation table (100 rpm). After washing with 0.05% Tween-20 in PBS, the wells were blocked with SuperBlock Protein with 0.05% Tween-20 (Pierce, USA) according to manufacturer's instruction, followed by six hour incubation at room temperature with sera and standards. Biotinylated goat anti-human midkine polyclonal antibodies (RnD Systems, USA) at concentration of 0.5 $\mu\text{g}/\text{ml}$ in T-PBS were added for overnight incubation at 4°C . Then, 0.2 $\mu\text{g}/\text{ml}$ of streptavidin conjugated with horseradish peroxidase (Jackson Immunoresearch, USA) in PBS with 0.05% Tween-20 was applied and allowed to react for 30 minutes at room temperature. The wells, after thorough wash with six changes of washing buffer, were next filled with substrate solution (1-Step ultra TMB-ELISA, Pierce, USA) for 30 minutes incubation in the dark. The enzymatic reaction was terminated by the addition of 2 M H_2SO_4 . The colour intensity was measured at 450 nm on a microplate colorimetric reader (Multiscan MS, Labsystems). As a standard, the authors used

recombinant human midkine (PeproTech, USA) in the concentration range of 10–2000 pg/ml, dissolved in ten times diluted human serum depleted of midkine by affinity chromatography on heparin-Sepharose (1 ml Hi-Trap Heparin HP column, Amersham Biosciences, Sweden). The depleted serum was collected and dialyzed against PBS with 0.05% Tween-20 and concentrated to the initial volume by centrifugation at 5,500 rpm, 4°C using vivaspin 20 ml concentrator with 3,000 MWCO PES membranes (Vivascience, Germany).

Calculation of Test Results

All samples were run in duplicates. The arithmetic means were calculated and the average of test sample blank absorbencies was subtracted from them. The resulting absorbencies of standards were plotted against standard concentration on log-log scale. The best-fitted reference curve was constructed by linear regression analysis. The measured absorbencies of examined samples were converted into midkine concentrations expressed in ng/ml and multiplied by serum dilution factor.

Statistical Analysis

Both studied groups were characterized by medians, means and standard deviations (SD). The upper normal range was established as average of midkine concentration in healthy controls + SD (82nd percentile) and incidence of hernia cases elevated above it was calculated. Non-parametric statistics (Mann-Whitney U test) was applied to evaluate the significance of observed differences between groups. A level of $p \leq 0.05$ was accepted as statistically significant.

Results

Serum midkine level was measured in apparently healthy blood donors. The median and mean values of serum midkine were 0.14 ng/ml and 0.221 ± 0.320 ng/ml, respectively, whereas in sera from hernia patients, midkine levels were higher with median and mean of 0.38 ng/ml and 0.652 ± 0.773 ng/ml, respectively. The upper normal range, based on midkine concentrations in sera from apparently healthy blood donors, calculated as mean + SD (82nd percentile) was 0.541 ng/ml. More than 43% of studied cases of rapture and only 5 out of 43 cases of controls were elevated above the upper limit of normal range. The observed significant differences ($p = 0.009$) in means

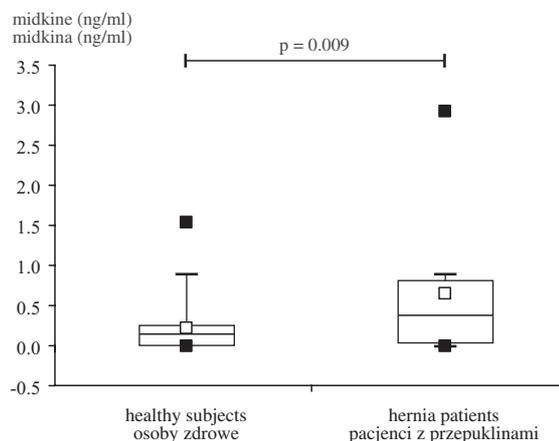


Fig. 1. Box chart of serum level of midkine in patients with hernia and healthy control group. The bottom and top of the box represent 25th and 75th percentile, respectively, whereas bottom and top closed squares minimal and maximal measured values, respectively. Open squares inside boxes represent mean values whereas inside bars median values. Bars outside boxes represent 5th and 95th percentile

Ryc. 1. Wykres ramkowy stężenia midkiny w surowicy krwi u pacjentów z przepuklinami oraz w grupie kontrolnej osób zdrowych. Górna i dolna podstawa ramki oznaczają, odpowiednio, dolny i górny kwadrant, a zamknięte kwadraty – wartości minimalną i maksymalną. Otwarte kwadraty wewnątrz ramek symbolizują wartość średnią, a kreski wewnątrz – medianę. Kreskami poza ramkami oznaczono 5. i 95. percentyl

between groups were evaluated with non-parametric Mann-Whitney U test at probability level of 95% (Fig. 1). Individual serum midkine values for both examined groups are presented in Figure 2.

Discussion

A group of hernia patients is often exploited as a reference in research on gastrointestinal malignancies as, in respect to biochemical profile, patients with rapture do not differ significantly from normal, healthy individuals [7]. The authors were, however, surprised by elevated midkine level in hernia group compared to the concentrations reported in the literature as midkine reference range [5, 6]. In this work the authors confronted midkine levels obtained for hernia patients, healthy in other respects, with those of blood donors, regarded as normal healthy individuals. Median midkine value obtained for blood donors group (0.14 ng/ml) was consistent with reference median value reported by Ikematsu et al. (0.154 ng/ml) [6]. Whereas median and mean values calculated for patients with rapture were significantly higher, although lower from those found in malignancies [4–6].

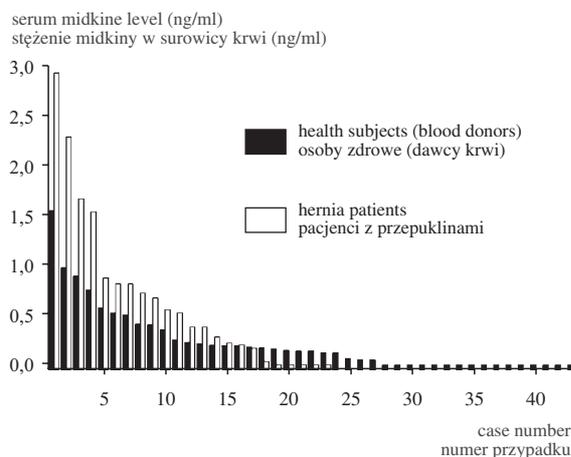


Fig. 2. Distribution of individual values of serum midkine concentrations in hernia patients and apparently healthy individuals

Ryc. 2. Rozkład pojedynczych wartości stężenia midkiny w surowicy krwi u pacjentów z przepuklinami i osób zdrowych

Muramatsu et al. [5] observed that in none of 67 studied cases the midkine level in serum of healthy subjects exceeded 0.6 ng/ml and in most cases it was undetectable. Moreover, in a more sensitive method of Ikematsu et al. [6] the highest obtained midkine concentration in a group of 135 normal individuals did not exceed 0.5 ng/ml. In presented method for immunodetection of serum midkine concentration, for a control group of blood donors, intermediate value of 0.54 ng/ml represented 82nd percentile, which is the upper limit of normal range. In 4 out of 43 cases in this group the limit of 0.6 ng/ml was exceeded whereas almost half of the studied cases of hernia was characterised by higher midkine concentrations (Figure 2). In only one case in controls the midkine level was higher than 1 ng/ml, while in hernia patients in 4 out of 23, with the highest obtained value of 2.92 ng/ml.

These observations suggest that in a case of studies on midkine, hernia patients should be rather excluded from control groups as they would raise the upper limit of normal range. Elevation of midkine concentration would not be surprising if blood was collected after surgery as overexpression of angiogenic factors is a part of wound healing process. However, hernia patients enrolled in

this study, were before the scheduled surgery and, in other respects, apparently healthy. Yet, other factors, such as obesity status has not been studied in this group of patients. Only this year a paper by Silha et al. [16] has been published in which elevated levels of some growth factors with angiogenic properties (VEGF-C and VEGF-D – Vascular Endothelial Growth Factor C and D) has been reported in sera from obese individuals. They suggest that obesity is accompanied by expansion of the capillaries in regional adipose depots and release of angiogenic factors. In this context BMI (Body Mass Index) should be taken into account in designing control groups in studies on angiogenic factors.

On the other hand, hernia is a condition in which more or less pronounced episodes of hypoxia can occur, depending on hernia location and severity [9] and hypoxia can induce expression of some angiogenic factors [10]. It has been found that in congenital diaphragmatic hernia, the expression of angiogenic factor VEGF-A was enhanced, in order to improve gas exchange by remodeling of vasculature [11]. The question, whether midkine can be regulated by hypoxia has been answered just recently, when Reynolds et al. [12] found that midkine expression in the respiratory epithelium was under control of hypoxia and hypoxia inducible factor 1 α (HIF-1 α).

Moreover, midkine is known to be overexpressed in esophageal malignancies [2, 13] and hiatal hernia is a condition associated with Barrett's esophagus [14], the precancerous lesion of esophageal adenocarcinoma. One cannot exclude that moderately elevated levels of midkine found in hernia may reflect some very early molecular changes occurring in sequence of events from normal squamous cell epithelium to Barrett's esophagus and, subsequently, to adenocarcinoma of esophagus. In addition, the size of hiatal hernia is considered the strongest predictor of esophagitis [15], in which condition midkine elevation could not be surprising as midkine is also a pro-inflammatory cytokine [2]. In this respect, it would be interesting to follow serum midkine concentration in subsequent stages of progression from normal squamous cell epithelium through esophagitis in gastroesophageal reflux disease (GERD), metaplasia, dysplasia and finally adenocarcinoma.

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