Genomic Polymorphism and Sepsis – Is There a Reason for Optimism?

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Abstract: There is no doubt that, in infectious disease, genetic predisposition plays a very important role in clinical outcome. Sepsis is a polygenic syndrome initiated by infection. A fact confounding the situation is that two factors – the macroorganism and the microorganism – are at play at the same time; hence of genotype effect must be assessed in light of their interaction. From a phylogenetic point of view, infectious disease is a companion of man throughout their life and its role in terms of function of the system of innate immunity is perceived as a beneficial one. However, the presence of a major antigen load by the infectious agent results in pathological responses at the levels of the macroorganism. Assessment of the severity of the inflammatory process on the basis of genetic predisposition is a most challenging issue. Genetic polymorphisms in the immune response to infection have been shown to be associated with clinical outcomes. The advancement of single nucleotide polymorphism (SNP) genotyping in basic genes – CD14, Toll like receptors, LBP, cytokines, cytokine receptors and coagulation factors have provided valuable information on the interaction of the macro and microorganisms. The understanding of the variation in genes and differences in response to infection may contribute to tailored diagnostic and therapeutic interventions with improved outcome in these patients.

Introduction

On April 14, 2003, the primary goal of the "Human Genome Project", that is, complete sequencing of the human genome, was accomplished. This was 50 years after the publication of a paper by Watson and Crick describing human DNA. The first report of the complete genome of a bacterium (*Haemophilus influenzae*) was published in 1995 [1]. In recent years, there has been an explosion of papers addressing the effect of genetic predisposition to the development and course of various conditions. There is little doubt the factors contributing to the course of a disease include, in addition to genetic predisposition, environmental factors and type of injury. Physicians now have the exciting opportunity to explore the pathogenesis of diseases at the level of cellular interaction. The advances made in biotechnology and bioinformatics offer new possibilities for predicting the susceptibility of an individual to a specific disease and clinical course. Sepsis as a polygenic and multifactorial syndrome poses a challenge for a search for such associations.

The role of the genome in sepsis

The genotype affects the incidence and severity of infectious disease. Numerous studies have demonstrated an association between the genomic variability of an individual with the incidence and outcome in inflammatory and infectious diseases. One of the first such studies was that conducted by Sorensen et al. who, while investigating the incidence of cancer and role of the genetic background, revealed

an association between infectious disease and the death of children whose biological parent died of infection at an age below 50 years (relative risk 5.8) [2]. Further evidence was furnished by studies with twins, designed to determine the incidence of tuberculosis, leprosy, poliomyelitis and hepatitis B [3]. Sepsis is a multifactorial complex syndrome with many non-specific symptoms [4]. This is partly due to the fact that inflammation – one of the main pathophysiological mechanisms – is not characteristic for sepsis only. Inflammation is encountered in a broad range of diseases not necessarily having an infectious aetiology. On the other hand, that's why one should logically assume the course of the disease is affected by the patient's genotype. A major role of genetic predisposition is based on the variability of the immune response of individuals without apparent abnormalities of the immune system.

Gene polymorphism and their study

The variability of the genome in the population does not seem to be very high, with an estimated 0.1% difference between individuals. These differences are referred to gene variation, polymorphism. By definition, a polymorphism is a difference in the gene sequence occurring with a frequency higher than 1% in the population. Polymorphisms may involve replacement of a nucleotide for another one, insertion of a nucleotide(s) or their deletion. Polymorphisms may occur both in the coding and non-coding genome regions. The frequency of polymorphisms in the non-coding regions is much higher compared with the coding ones. This is due to the higher degree of preservation of exons to assure the functionality of gene products and the fact that any change in sequence can be considered a potential mutation. However, even polymorphisms and mutations in non-coding regions may exert a dramatic effect on phenotype presentations. They are mostly changes interfering with the structure and process of transcription and gene expression. A specific type of a polymorphism often in diagnosis because of its high informative value are microsatellite markers, i.e., a variable repetition of DNA sequences [e.g., $(CA)_n$; n = number of repetitions].

The most common polymorphism involves the replacement of a single nucleotide base (single nucleotide polymorphism, SNP). This polymorphism occurs in approximately 500–1000 DNA bases. This type of a polymorphism in the coding regions presents itself either as the creation of a "de novo" site where transcription ends or as a change in the sequence resulting in a change of the respective amino acid. There has been an effort to clarify the potential causal relationship between these changes and the development and course of diseases to modulate a patient's response to administration of drugs [5]. The association between the genome and disease cannot be perceived as an isolated issue, not related to the whole of cell biology. In addition to the genome, other dynamic processes including transcriptomics (messenger RNA), proteomics (proteins), physiomics (communication networks and signalling pathways) and biotics (cell

phenotype) influences the function of cells and organ [6]. As a result, the response by an individual to injury is only partly dependent on their genotype.

Several molecular genetic approaches can be employed in the search for polymorphisms and mutations. The conventional method most widely used in Mendelian syndrome (i.e., a model assuming a single gene-single disease and, consequently a single mutation/polymorphism – a single genotype) is linkage analysis. This analysis is based on the genotyping of microsatellite markers with high heterogeneity in patients' families. Provided linkage analysis has demonstrated the linkage of a microsatellite marker with a disease, the search begins, in the given region, for a mutation or a polymorphism causal for the phenotype in the family in question. However, this approach is not appropriate for such a polygenous and complex syndrome that sepsis is.

A more appropriate approach is what is called the associated study design (a group type of a study), whereby polymorphisms of genes playing a potential role in the pathogenesis of the disease are selected. This type of a study must be conducted using large patient cohorts as the causality of a polymorphism is the result of statistical analysis of frequency of polymorphism in question in populations of patients and healthy individuals. Association studies reveal the presence of the given alleles in patients and in a control population. These studies are more effective in many respects, particularly with diseases, which are the result of modulation of the body's response by numerous genes and the environment. However, they are also fraught with some pitfalls related to their interpretation. An inherent error due to the nature of the study is the demonstration of an association between a polymorphism and a disease in cases where no such association is in fact present, that is, a polymorphism consistent with the features of a disease may occur (while localized, unlike the causal one, on a completely different chromosome or be part of a different group of genes or a gene), resulting in false interpretation of its causality. The study may then erroneously show an increased population- or race-specific incidence of a polymorphism and disease although no association is actually present. This is the most challenging aspect of association studies in identifying causality. To exclude these errors, association studies are complemented with the transmission disequilibrium test (TDT) designed to assess the presence of potential causal polymorphisms in a preceding generation. The test is based on the assumption that the phenotype of a patient is the result of the presence of a certain allele(s) so parents heterozygous for this allele(s) must have passed it on to their offspring. The prevalent presence of a certain allele(s) in patients makes the association of between this allele(s) and disease likely.

Errors in interpretation can also be reduced by using appropriately sized groups of patients and controls. Long and Langley have suggested at least 500 subjects are required in studies of this type to stand an 80% chance of revealing a polymorphism association in the locus in question.

The techniques employed in the search for polymorphisms are adapted to meet the requirements of individual screening programs. Traditional techniques of molecular genetics (PCR, electrophoretic separation or FRET analysis and others) can be used when assessing a small number of polymorphism. Studies designed to investigate numerous polymorphisms make use of novel technologies such as DNA microarrays. This is essentially a hybridization technique using a fixed matrix allowing polymorphism detection or semiquantitative determination of expression of genes involved in the given disease. The method allows furnishing direct evidence about an association between an individual's genotype and their susceptibility to the given disease [7].

Polymorphisms of innate immune receptors

Sepsis is a systemic inflammatory response of the body to infection. As regards aetiology, bacterial, viral, fungal, and parasitic agents have all been implicated. In Gram-negative infection, the underlying structure of its pathogenicity is endotoxin or lipid A. Endotoxin-like effects have been documented with Gram-positive bacterial structures, i.e., teichoic and lipoteichoic acids, capsular lipopolysaccharide (LPS) and group-specific carbohydrates. Induction of the immune response in Gram-negative infection requiring the binding of LPS to the monocyte phagocyte receptor, CD14 molecule, which is a 53 kDa glycoprotein receptor expressed on the surface of myelomonocyte cells. In addition to this cellular form, there are two soluble forms CD14 (sCD14) inducing the development of an inflammatory cascade through the binding to LPS. The protein immediately responsible for the binding of LPS to CD14 is liver-derived lipopolysaccharide-binding protein (LBP) present in the plasma [8]. A functionally related is bactericidial permeability increasing protein (BPI) produced by polymorphonuclear cell. BPI is cytotoxic to Gram-negative bacteria but inhibits LPS binding to CD14-positive monocytes [9].

The association between CD14 polymorphism, LBP, and BPI in sepsis patients was investigated. Separate studies reported increased sCD14 levels in patients with Gram-negative and Gram-positive septic shock as well as their increased mortality [10, 11]. In a German study, gene polymorphism of the CD14 promoter on position -159 (C-159) associated with increased levels of soluble and membrane CD14, but not with increased mortality [12]. By contrast, a French study reported an increased incidence of septic shock in patients homozygous for the T allele. A TT genotype was associated with increased risk for case fatality (odds ratio of 5.3) independent of the allele for tumor-necrosis factor [13]. The polymorphism of BPI and LBP (Lys216Glu, Pst1(T $\rightarrow$ C) in intron 5 and G535C for BPI; Cys98Gly and Pro436Leu for LBP) was not significantly different between sepsis patients and controls [14]. A Czech study evaluated the role of genetic polymorphisms of the bactericidal permeability increasing protein (BPI) in pediatric patients with sepsis. A statistically significant predisposition to Gram-negative sepsis in patients carrying the BPI Taq GG variant together with the BPI 216 AG or GG variant was revealed [15].

An important family of receptors involved in recognizing alien structures of bacterial, viral, and parasitic origin is that of Toll-like receptors (TLRs) [16]. TLRs play a central role in the innate immune response to infection through the recognition of different bacterial antigens. TLR4 is crucial for the recognition of lipopolysaccharide, TLR2 is essential in the recognition of Gram-positive bacterial components – i.e., lipoteichoic acid or peptidoglycan. Using an experimental model, mutations in the TLR4 gene (Pro712His) as well as TLR4 deletion have been shown result in hyporesponsiveness to purified lipopolysaccharide, but also increased susceptibility to Gram-negative infection [17]. Human studies have identified a mutation on position 299 (Asp299Gly) and Thr399Ile on the TRL4 extracellular domain with decreased reactivity to lipopolysaccharide administration [18]. A French study did not demonstrate a different prevalence of these mutations in sepsis patients and controls. By contrast, the Asp299Gly mutation was present in five patients in septic shock as opposed to none in the control group [19]. A study by Read et al. did not show an association between Asp299Gly TLR4 polymorphism and susceptibility or severity of meningococcal infection [20]. Smirnov et al. reported that 7.5% of cases of meningococcal sepsis in a population of British patients were due to TLR4 mutation [21]. These differences again highlight the large variability in the phenotype of sepsis.

A defective TLR2 gene predicts increased susceptibility to infection by Gram-positive bacteria [22]. A potential association between the Arg753Gln TLR2 mutation and septic shock was suggested by Lorenz et al. [23]. Very recently, Wurfel et al [24] showed that hypermorphic genetic variation in TLR1 is associated with increased susceptibility to organ dysfunction, death, and gram-positive infection in sepsis.

Polymorphism of cytokines and their receptors

A key role in the pathogenesis of sepsis is played by the balance or imbalance of pro- and anti-inflammatory cytokines. The pro-inflammatory cytokines include tumor-necrosis factor-α (TNF-α), IL-1 (IL-1), interleukin-6 (IL-6), while those with anti-inflammatory activity include interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13) and transforming growth factor β [25].

TNF-α is one of the best characterized cytokines in the pathogenesis of sepsis. Besides the deleterious of increased TNF-α synthesis, its inadequate production may also have adverse effects [26]. Complete TNF-α inhibition resulted in high death rates in experimental animals. When using recombinant soluble TNF receptor (rsTNFR), the mortality of patients receiving high doses of rsTNFR was higher compared with placebo-treated patients [27]. Two TNF locus polymorphisms associated with TNF production have been identified to date. The bi-allelic polymorphism on position 308 (G308–TNF1 allele) and (A 308-TNF2 allele) has been related to increased TNF-α production *in vivo* [28]. Investigations of these polymorphisms in sepsis patients have not yielded consistent results.

A German study failed to demonstrate an association between a TNF2 polymorphism and severe sepsis [29]. Tang and co-workers likewise did not demonstrate a predisposition of the TNF2 allele to the development of septic shock or increased mortality. However, in patients heterozygous for TNF2 polymorphism developing septic shock, both serum TNF-α levels and mortality were higher compared with TNF1 homozygous patients [30]. Similar conclusions were reported by Mira et al. [31]. By contrast, a US study shown that being a carrier of the TNF1 haplotype was protective against the development of sepsis [32]. In a German study, a TNF-β polymorphism (TNFB2 homozygotes) was associated with increased mortality and higher TNF-α levels in patients undergoing surgery or in trauma patients [33, 34]. It is now generally believed that TNFB2 polymorphism is in linkage imbalance with TNF1 polymorphism, suggesting a potential causal role for sepsis.

Gene polymorphism has also been demonstrated for other cytokines including IL-1, IL-1β, and IL-1 receptor antagonist (IL-1RA). Two bi-allelic polymorphisms have been reported for the IL-1β gene, AvaI polymorphism on position -511 and TaqI polymorphism on position +3953 [35, 36]. The polymorphism of the IL-1RA gene (A2 allele in intron 2) is associated with enhanced IL-1RA production following *in vitro* stimulation [37, 38]. The presence of these polymorphisms in sepsis has been explored in several studies. Fang et al. showed a higher frequency of the IL-1Ra A2 allele in patients with severe sepsis compared with a control group of healthy individuals [39]. An association with clinical outcome and the presence of IL-1β or the IL-1Ra allele was not found. The Chinese study by Ma et al. did demonstrate an association between the risk for developing sepsis and IL-1RN2 polymorphism; the RN2 and RN2/2 genotypes was significantly more frequent in the group of sepsis patients compared with controls ($p<0.01$ vs. 0.05). In addition, the A2/2, B2/2 and RN2/2 genotypes predicted higher mortality in sepsis patients compared with the A1/1, B1/1 or RN1/1 genotypes (mortality rates of 70–80% vs. 0–13%) [40]. Arnalich et al., while demonstrating an association between mortality and presence of IL-1RaA2 homozygocity in a group of patients with severe sepsis, they did not document an association between the risk for developing sepsis and the presence of the polymorphism [41]. These results suggest a potential benefit of detecting IL-1 gene family polymorphisms for the prediction of sepsis development and mortality; however, larger studies related to different ethnic groups are warranted.

Another important pleiotropic cytokine in the pathogenesis of sepsis is IL-6. IL-6 acts as an activation stimulus for T-lymphocytes and induces antibody production by B-lymphocytes together with differentiation cytotoxic T-lymphocytes. IL-6 is a potent inducer of the acute phase proteins C-reactive protein, fibrinogen and serum protein A. the role of IL-6 in the pathogenesis of sepsis is not fully understood yet [42]. The effects of IL-6 are both proinflammatory (coagulation system activation) and anti-inflammatory at the systemic and compartment levels

[43]. Endogenous IL-6 promotes macroorganism defences similar to TNF [44]. In their multicenter study, Reinhart et al. have shown that IL-6 levels >1000 pg/ml predict significantly higher mortality of patients [45]. A polymorphism on position -174 in the IL-6 promoter region 6 (G-174C) has been identified. Presence of the C allele has been linked to lower IL-6 production. A German study [46] did not demonstrate significant differences in the frequencies of the polymorphism or genotype between patients with and without sepsis. GG homozygocity was associated with lower mortality in the group of sepsis patients. An association between the genotype and systemic IL-6 levels has not been shown. These conclusions were subsequently confirmed by a study in trauma patients [47]. A Czech study investigated two IL-6 gene polymorphisms (G-174>C and G-572>C). The IL-6 gene polymorphisms G-174>C and G-572>C could be predictors of the risk for developing and/or the predictors of the severity of sepsis in children [48].

Interferon gamma (IFN-γ) produced by T-lymphocytes and natural killers (NK cells) shows immunoregulatory activity in relation to HLA-DR Class II expression, and IL-1 tumor-necrosis factor production [49]. Patients after major surgery have been shown to have significantly reduced interferon-γ production [50]. Sepsis will make this insufficiency still worse and the condition may result in functional monocyte inactivity and decreased expression of MHC Class II traits. Interferon-γ exerts inhibitory effects on Il-10 production by monocytes, inhibits prostaglandin E2 release and stimulates TNF and IL-1 production by monocytes. In this context, it seems to be a good candidate for immunomodulatory therapy in these patients [51]. Polymorphism of the IFN-γ receptor (IFN-γR1) and the IFN-γ gene has been identified in trauma patients subsequently developing infectious complications [52, 53]. Here again, further studies are needed to confirm the results obtained to date.

IL-10 is a potent anti-inflammatory cytokine involved in the suppression of innate and adaptive immune responses. IL-10 plays a very important role in the process of induction of immunoparalysis. IL-10 suppresses the expression of MHC II class molecules and the production of proinflammatory cytokines and chemokines by activated monocytes [54]. There are three polymorphisms in the promoter region of the IL-10 gene occurring at -1082, -819, and 592. Lowe et al demonstrated that the -592 A/A genotype is associated with a decrease in IL-10 production and an increase in mortality in sepsis patients (55). Schaaf et al showed an increase in IL-10 levels in sepsis patients who were homozygous for the -1082 G/G genotype when compared with the A/A or A/G genotypes [56].

Polymorphism of endothelial dysfunction factors

A characteristic feature of sepsis is inadequate activation of the inflammatory response with impaired vascular tone, inadequate activation of leukocytes, and impaired coagulation homeostasis. Production of coagulation factors is likewise

disturbed [57]. The cytokines produced in sepsis patients activate their coagulation system. The key role in the coagulation cascade is played by tissue factor (TF) activated by TNF-α, IL-2, and IL-6 (58). Cytokine action results in reduced fibrinolysis and decreases in protein C and antithrombin III levels. Activated protein C inhibits factors Va and VIIa, and plasminogen activator inhibitor-1 (PAI-1). Antithrombin III inhibits the extrinsic coagulation factors Xa, XIa, IIa, and plasmin. A progressive procoagulation state enhances the inflammatory response in sepsis [59]. Meningococcal sepsis is associated with decreased thrombomodulin and endothelial protein C receptor expression [60]. High PAI-1 levels are associated with a clinical prognosis of sepsis [61, 62]. A polymorphism of the PAI-1 gene promoter region (4G/5G) associated with increased plasma PAI-1 levels has been reported [63].

A group studying meningococcal disease compared a group of patients with meningococcal infection and healthy controls. Patients with the 4G/4C genotype had significantly higher plasma PAI-1 levels and were at increased risk of death compared with patients with the 4G/5G or 5G/5G genotype [64]. The same conclusions were made by the authors of a Danish study [65]. A recent study [66] confirmed earlier results. In a cohort of 510 of paediatric patients, its authors documented higher death rates of patients with the PAI-1 4G/4G genotype (28.4%) compared with the 4G/5G and 5G/5G genotypes (14.9%; p=0.005; RR 1.9, CI 1.2–3.0). Surviving patients with the 4G/4G genotype also had significantly higher rates of vascular complications. A study in trauma patients demonstrated a correlation between the 4G/4G genotype and higher levels of IL-1, TNF-α, and PAI-1 compared with healthy volunteers, together with a poorer clinical prognosis [67].

Pharmacogenomics and sepsis

In addition to the above factors, an important role in the clinical outcome of sepsis patients is played by pharmacogenomics. This is closely related to different ability of individuals to metabolize drugs depending on their sex, age, ethnicity, and co-existing conditions. It is clear today that genetic predisposition plays a crucial role in one's ability to metabolize drugs [62]. In the US, 106,000 patients die every year from serious reactions to ingestion of drugs. Recently, it has been reported that 60% of drugs causing serious unwanted effects are metabolized by at least one enzyme with the presence of an allele affecting drug metabolism [63]. Gene polymorphisms have been identified in many enzymes involved in the metabolism of drugs [64]. The ever increasing importance of pharmacogenomics in sepsis is exemplified by the PROWESS trial and application of knowledge derived from pharmacogenomics. Russell et al. studied the genetic markers of patients treated by activated protein C (Xigris). They investigated two candidate genes, i.e., the protein C gene (or its polymorphism 9912) and the PAI-1 gene. In the case of the protein C gene, there was no difference in the fatality of patients with the TT

genotype receiving Xigris or standard therapy. In patients with only one T allele copy (e.g., CC or CT), the administration of Xigris resulted in a reduction in mortality from 50% to 30%.

Conclusion

Determination of genetic predisposition in sepsis has become an integral part of current clinical research in the critically ill. New techniques and sophisticated computer technology have contributed to major advances in this line of research. Despite the positive or negative associations between polymorphism and outcome that were identified in critical illness, the confidence in such conclusion is often low due to problems with experimental design, statistical analysis, study size, power, and replication. Results obtained to date provide a reason for cautious optimism in assessing the role of these tools in the diagnosis, pathogenesis, and potential treatment of sepsis. The complexity of changes in a polygenic disease, which sepsis is, and the intricacies of diagnostic and analytical procedures make it clearly imperative to launch large national or multinational studies and to concentrate clinical research into selected centres with established diagnostic capabilities using molecular biology.

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