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T-cell epitope identification strategies

The first step in the development of anti-tumour peptide vaccines

Bachelor thesis by

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Abstract

Anti-tumour peptide vaccination is an upcoming variant of immunotherapy against malignancies. These vaccinations succeed in creating reactive T-cells, but often do not lead to tumour shrinkage. Because of this lack of clinical benefits improvement of these vaccines is needed. Crucial for the success of therapeutic cancer vaccines is the induction of a functional immune response. The best scenario is the creation of a broad immune response. Therefore identification of epitopes that can be presented by the MHC molecules and induce a good immune response is needed. A good epitope has high affinity for the MHC complex, is immunogenic and tumour specific. The identification of epitopes started in the 90's. However, there are still a lot of epitopes that need to be discovered. With all the developments in technology, high-throughput strategies for epitope identification have been developed. These strategies are also known as 'reverse immunology'. This innovation led to database-driven research into new epitopes. Instead of starting at the epitope and revealing its amino acid sequence, reverse immunology starts with predicting epitopes and confirming them *in vitro* or *in vivo* at the end of the process. *In silico* predictions of epitopes are made with algorithms. A large number of epitope prediction databases are available on the internet. Every database has its advantages and drawbacks since they all use their own algorithms. The goal in this paper is to give an overview of reverse immunology epitope identification strategies and presenting the best manner of epitope identification.

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1. Introduction

The human immune system can be divided into the innate immune system and the adaptive immune system. The latter is responsible for the specific response to antigens, presented by infected cells or by specialised antigen presenting cells (APC)[1]. The most important components of the adaptive response are the B- and T-cells. These cells are capable of rearranging their genes, and thereby create a very specific immune response[2,3]. This specific response is responsible for protection against infected cells, and for protection against malignant cells.

Overall the immune system plays a crucial role in the defence against cancer, which can be used to develop novel therapies; immunotherapy[4,5]. One of these therapies aims to induce a tumour-specific immune response through peptide vaccination[6]. Research shows that patients with a pre-existing immune response in general respond better on cancer treatments. This suggests that anti-tumour peptide vaccination can be a very successful (adjuvant) therapy[7,8]. Research into anti-tumour peptide vaccination has shown the possibility to generate tumour-specific $CD8^+$ cytotoxic T-lymphocytes (CTL), but real clinical benefits, like tumour shrinkage, with use of these CTLs have not been accomplished yet[6,8-10]. One factor in success is the use of correct peptides. Therefore suitable epitopes should be identified and carefully selected for peptide vaccination.

For a successful immune response T-cells need to recognise antigens presented by the infected or malignant cell[1,11]. T-cells can recognise target cells with their T-cell receptor (TCR). TCRs recognise (non-self) peptides presented by antigen presenting cells (APC) or tumour cells on the major histocompatibility complexes (MHC). APCs can prime naïve T-cells when presenting a tumour peptide. Priming leads to development of tumour-specific CD8+ cytotoxic T-cells (CTL). These CTLs can be activated when they recognise their specific peptide on the tumour cells. In this way CTLs are activated to kill tumour cells. Thus, recognition of MHC presented peptides is of great importance for a successful immune response. This recognition is accomplished through the TCR recognising a specific epitope of the presented peptide[3,12,13].

The working of an anti-tumour peptide vaccine is dependent on the injected tumour specific epitopes[14]. It is possible to inject the whole tumour antigen. In that case epitope identification and selection is not needed. This full-length protein contains multiple epitopes for T-cell recognition and differences in MHC alleles between patients are not of influence[15]. However, a broad T-cell response against a number of epitopes can contribute to a successful anti-tumour response. With a full-length peptide vaccination one single immunodominant epitope can overrule subdominant eptiopes[16]. Another option can be to use peptides containing the suitable epitopes. In this case epitope identification is necessary[17]. However, when the protein is 'cut' in the wrong places the peptides might not consist of the right epitopes. The generation of overlapping peptides covering the full protein can be a solution. Moreover, competition between dominant and subdominant epitopes can occur, which still will not lead to a broad T-cell response[19].

Altogether, the success of anti-tumour vaccination is greatly influenced by the epitopes that are used and in how they are administered. Thus, it is important to identify T-cell epitopes. However there

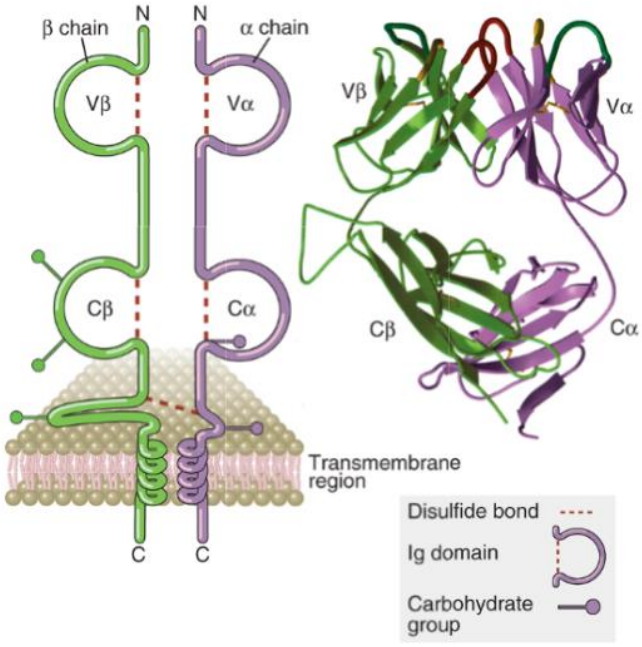
are many ways possible to try and identify new epitopes. Epitope identification strategies started to be developed in the 1990's, these strategies are now referred to as 'direct immunology'[17,20]. More efficient and high-throughput strategies are in development; the 'reverse immunology'[21,22]. Here an overview will be given of currently available strategies for identification of epitopes that are capable of producing a functional $CD8^+$ and/or $CD4^+$ T-cell response. Verification of the predicted epitopes is also an important step in this process. This paper will conclude with the best strategy for epitope identification.

2. T-cell activation

2.1 Characteristics of the T-cell receptor

The T-cell receptor (TCR) is the machinery for T-cells to recognise antigens, which can be found on both CD4+ and CD8+ T-cells. The TCR is the same for both, except for the hypervariable region. The TCR is heterodimeric, since it is built out of an α- and β-chain. Both chains have an Ig-like N-terminal variable domain (V), and an Ig-like constant domain (C). The chains are connected through disulphide bridges[1,21]. This is represented in figure 1.

When traveling through the thymus, T-cells mature. Their specificity is accomplished by gene rearrangement of both the alpha and beta chain. These somatic rearrangements take place in the V, J and D gene regions[23]. This results in a unique hypervariable region on each mature T-cell when they leave the thymus. In one human being this is estimated to result in $2{,}5\times10^{7}$ different TCRs[24]. They are positively selected to be functional, and negative selection takes place for T-cells that respond to auto-antigens. This should prevent the generation of auto-responsive T-cells [25,26]. After this journey, naïve CD4+ and CD8+ T-cells enter the bloodstream[12].



Figuur 1 T-cell receptor. Schematic diagram of the T-cell receptor with α- and β-chain and their constant (C) and variable (V) domains. On the right the 3D structure is shown. Binding with the peptide-MHC complex is facilitated trough the hypervariable stemloops on the upper side of TCR. *(Modified from Abbas, Cellular and molecular Immunology 7th edition [1])*

2.2 Characteristics of the MHC molecule

Genes for the human variant of the MHC complex, called HLA, can be found on chromosome 6. HLA genes are highly polymorphic and there is great variety between human in HLA haplotypes[27]. Furthermore we distinguish between HLA class I and HLA class II molecules.
HLA-I is presented by all human cells with a nucleus[28]. HLA class I molecules present fragments of 8-12 amino acids, with the best binding capacity to a nonamere[29,30]. It is capable of activation CD8+ T-cells when presenting the right epitope. Even though HLA class I is presented on all nucleated cells,

the first priming of a naive CD8+ T-cell is required to be done by a specialised APC. Re-stimulation generated epitope specific CTLs can then be performed by all cells with HLA-I bound to the right peptide[21].

The presentation of peptides by the HLA complex is accomplished through a multiple-step pathway. First proteins in the cells collide with the proteasome, which is an ubiquitous enzyme and degrades the protein into smaller peptides. Some of these generated peptides will be presented by the HLA-I molecule. First they need to be transported to the ER with help of the transporter for antigen processing (TAP). Together with other molecules involved, the peptide can be loaded into the HLA-I peptide loading complex[31]. The proteasome degradation and peptide binding steps are highly sequence specific[32]. Only a small amount of generated peptide can be bound and presented by the HLA-I molecule[33,34]. This process of antigen presentation is described in figure 2.

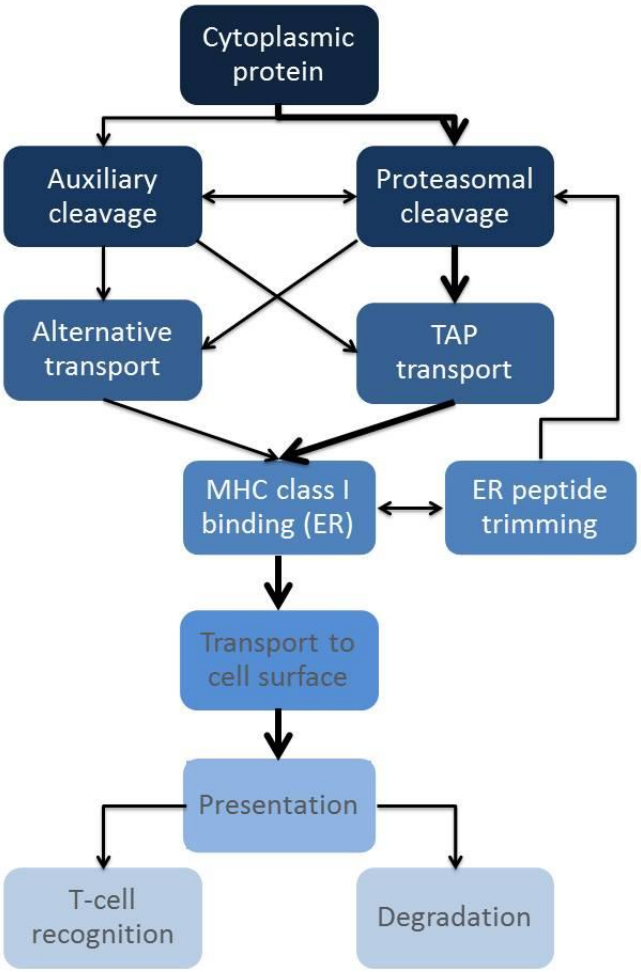


Figure 2 Antigen processing and presentation by HLA-I. Pathway of protein, first being cleavage and transported through multiple mechanisms. This is followed by the MHC class binding in the ER, with possible peptide trimming. The MHC-peptide complex is then ready to be transported to the cell surface for presentation. It is now possible for a CD8+ T-cell to recognise the presented epitope with the TCR. Otherwise the complex is taken back into the cell and degraded. (*Modified from Petrovsky & Brusic, 2004)*[32]

HLA-II processing is done in similar fashion. HLA class II molecules are capable of presenting longer fragments from 12-25 amino acids, since the binding groove is opened from two sides[29,35].

However the end result is accomplished through another pathway with its own enzymes, proteins and chaperones[36]. Moreover, HLA-II is responsible for stimulation of the CD4+ T-cells rather than the CD8+ T-cells. Another important difference is that presentation through HLA-II is restricted to antigen presenting cells (APC). For example dendritic cells and B-cells[3,12,28].

2.3 HLA peptide presentation and TCR recognition

The peptide-MHC complex (pMHC) is ready to be recognised by the TCR. This is shown in figure 3. The MHC molecules are only stable with a fitting peptide buried away in the bindingroove[36,37]. Recognition of the pMHC complex is highly dependent on the binding capacity of the TCR to the complex. The three complementarity-determining regions (CDR) are of importance for this. Where CDR1 and CDR2 have found to be important in binding of the HLA complex itself, CDR3 is responsible for the interaction with the bound protein and therefore important for epitope recognition[38].

Another important component in the interaction between pMHC and TCR are the co-receptors. CD8+ T-cells can recognise HLA class I molecules with bound peptide, in which CD8 functions as co-receptor[39]. Activation of these cells leads to the CD8+ cytotoxic T-cells (CTL) and clonal expansion. CTLs are capable of producing toxic enzymes and will lead to lysis and apoptosis of the infected or malignant cell[12]. Furthermore HLA class II molecules are recognised by the CD4+ T-cells[39]. These cells are then stimulated to form T-helper cells. These are important in the stimulation of CTLs for proliferation, expansion and creation of long-lived memory cells[40]. For a long lasting and successful immune response activation of $CD4^+$ cells and $CD8^+$ CTLs is necessary[40].

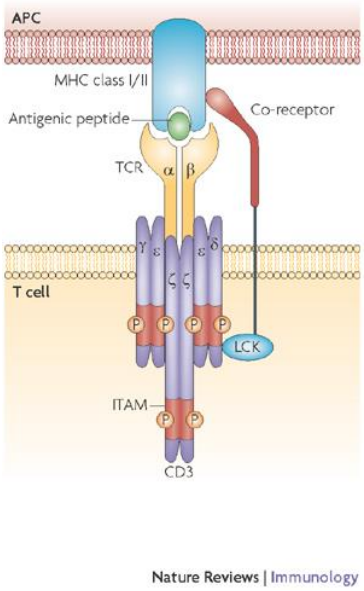


Figure 3. HLA-TCR interactions. Recognition of the pMHC by the TCR. Co-receptor helps the recognition process. This can be CD4, which recognises the MHC class II molecules. Otherwise the co-receptor can be CD8, which recognises MHC class I molecules. This interaction is capable of activation of the T-cell and the start of the immune response. (*Figure edited from Gascoigne N.R.J., 2007)*

3. Appropriate peptide selection

3.1 Tumour-associated antigens as vaccination peptides

An important step in anti-tumour peptide vaccine development is the choice of peptide. The peptide should create an immune response against the tumour alone. This can be achieved by priming the immune system with the right epitopes. This leads to a specific and functional response to the tumour cells expressing these epitopes. However, tumour cells are malignant cells that arise from the host itself. Therefore the antigens expressed by a tumour cell can be expressed by normal healthy cells as well[41]. Research got closer to accomplishing this with the discovery of the tumour-associated antigens (TAA). Peptide-derived variants of the TAAs are now commonly used in anti-tumour peptide vaccination trials[8]. TAAs can be divided into categories, all of them with benefits and drawbacks in the use as a peptide vaccine.

(1) The Cancer-testis (CT) antigens are antigens found in a variety of tumours, so not specific for one type of cancer. They can also be found in normal cells, but only in the testis or sometimes placenta[8,42]. Since they are found in a variety of tumours it can be applied in a broad manner, making it financially attractive[43]. More specific TAAs are (2) neoantigens. These are antigens that arise because of mutation in the tumour cells, which makes them very specific and a good target for vaccination. No normal cells will express this antigen[8,42]. However, they are also patiënt-specific, which makes the production of vaccines targeting neoantigens expensive[8,42,44,45]. (3) Linage-specific differentiation antigens are antigens that can be found in the tumour cells as well as in the linage from which the tumour arose. So they are not exclusively tumour-specific. Even though there is auto-expression of this antigen, successes have been made[8]. For example in solid breast tumours and melanomas[8,42,44]. Another option is (4) auto antigens that are being overexpressed in tumour cells. The same TAA can be found in a variety of cancers. There could be a chance of auto-immunity or no response because negative selection of T-cells in the thymus. However research shows possible good results from studies with overexpressed auto antigens[8].

In conclusion there is great variety in types of TAAs, all with their advantages and disadvantages.

3.2 Peptide length influences T-cell priming

One of the most important properties of a peptide which determines the success of a vaccine, is the length of the peptide[46]. Over the years research points out that clinical success of vaccines with short peptides only containing a $CD8^+$ T-cell epitope is moderate, because of poor immunogenicity. It seems that the activation of the $CD8^+$ CTLs has been the focus of anti-tumour vaccines, as these cells are capable of direct killing of tumour cells[47]. However, peptides containing epitopes capable of activating $CD8^+$ CTLs and $CD4^+$ T-helper (Th) cells as well can generate a better immune response[48]. The CD4+ Th cells play an essential role in the success of the immune response. This is for stimulation and proliferation of CTLs and especially important in the generation of CD8+ memory cells[49].

A short peptide representing the epitope necessary for TCR recognition is a tempting choice for vaccination, since it is ready to bind the MHC molecule. However research shows that the use of short peptides often leads to the induction of tolerance[47,50]. Yet, with the right adjuvant and vaccination intervals, these short peptides possibly could be capable of inducing an immunological

response[51]. Also, the short peptides are ready to be bound by MHC right away, while the long peptides still need to be processed in the right manner to be effective[43].

In general better successes are made with long peptide vaccination. One of these successes has been shown by *Zweveling et al.* as they proved the benefit of long peptide vaccination over short peptide vaccination[52]. This success can be explained because of the stimulation of $CD8^+$ and $CD4^+$ T-cells rather than $CD8^+$ T-cells alone. This was achieved with epitope-linkage; the use of a long protein, consisting of an epitope for $CD8^+$ as well as $CD4^+$ T-cells[48]. The success of this epitope-linkage strategy can be explained with help of the dendritic cell (DC). DCs are capable of presenting molecules through MHC-I and MHC-II, which is essential and known as cross-presentation. Short peptides are HLA class I restricted, so presentation can occur in all nucleated cells. Because of the high number of cells presenting the antigen, tolerance can be induced[53]. However, the use of long peptides or proteins can prevent this since these can be ingested only by DCs and presented through the MHC-I and MHC-II of this DCs. Cross-presentation will enhance the immune response, because both $CD4^+$ and $CD8^+$ T-cells are activated[44,54].

3.3 Immunodominance and epitope competition

When vaccinating with one specific peptide that leads to an effective immune response, malignant cells that do not express the targeted antigen have a good chance of survival. Theoretically it is possible to kill all tumour cells because of a phenomenon called epitope spreading; the immune response against one epitope activates an immune response against other epitopes as well[55,56]. Since this phenomenon does not always occur, it is not a safe option to depend on for vaccination.

The wanted scenario is to generate a broad immune response. An immune response against multiple epitopes could be generated when administering the whole antigen, harbouring multiple epitopes. However, an antigen contains dominant and subdominant epitopes[57]. When the dominant epitope overrules the subdominant epitopes, a broad immune response is still not accomplished. This can be solved by administering multiple peptides, thus multiple epitopes in one vaccination. However a concern in multiple peptide vaccination is the competition for MHC binding. Since one peptide can have higher affinity than others, this can decrease MHC presentation of the peptides with lower affinity[50]. One solution to prevent or limit competition is the use of multiple injections, administered at the same time at different sites off the body. Although there is nothing wrong with this method, it does create discomfort when the number of peptides to administer is 4, 5 or even higher. Multiple studies have been performed to investigate the effect of competing peptides with different results. Some research shows that a mix of multiple peptides can affect each other and decrease vaccination success[58]. However, other researches show multiple peptide vaccination to be successful[55]. There is not always inhibition of low affinity peptides when administered together with more dominant peptides[59]. Thus, the influence peptides can have on each should be determined per vaccine for all the peptides in the desired vaccine.

4. Epitope identification strategies

4.1 Direct and Reverse immunology

Identification of epitopes within proteins is necessary to know what amino acid sequence is capable of activating T-cells. The identification of epitopes started with *Boon et al*[17]*.* His manner of epitope identification is usually referred to as 'genetic immunology'. The strategy involves isolated CTLs that have infiltrated the tumour of the patient. These need to be mixed with cells transfected with a cDNA library, created with the DNA of tumour cells expressing antigens of interest. When the CTLs are stimulated, clonal expansion takes place. This serves as selection of the cells that are capable of activating CTLs is possible. The next step was to retrieve the gene of interest from these cells and sequence this, resulting in an epitope sequence[20,60]. The strategy was successful since it led to the discovery of a lot of identified epitopes. However, the strategy is very labour intensive and time consuming[41].

New methods are used these days. One example is 'direct immunology'. This method focuses on the elution of tumour associated peptides from patiënt-derived cells. The isolated peptides can then be sequenced and subjected to mass spectrometry[61]. However the most commonly used method is 'reverse immunology'[61].

Reverse immunology identification starts with *in silico* predictions. Then binding affinity needs to be checked *in vitro* and immunogenicity can be determined *in vivo.* Because of *in silico* computer predictions there is no need for patient material. This new high-throughput strategy can be explained in four easy steps. Starting with the selection of possible genes expressing a peptide of interest, best to be tumour-restricted which would make it more specific. The epitopes are predicted with computer software of choice. The results are just a prediction, so this needs to be confirmed *in vitro*. This confirmation is performed by checking proteasome digestion and performing a TAP and MHC binding affinity assay[21]. The last step is testing immunogenicity, which can be performed either in vitro, with T-cell expansion, or *in vivo,* in an animal model[62]. If an epitope is successfully tested in all stages, peptides can be synthesised for a peptide vaccination trial[21,63]. This pathway of reverse immunology is represented in figure 4.

4.2 *In silico;* computer based epitope predictions

As the pipeline of figure 4 suggests, reverse immunology starts with the epitope, which is validated in multiple steps, and ends with tumour CTLs actually recognising and responding to this epitope. This could be regarded, like the name suggests, as a reverse manner of identification. Developments in technology lead to better ways of data management and high-throughput screening methods. The reverse immunology strategy for epitope detection is driven by the large amounts of available data, instead of the 'normal' hypothesis-driven research[64]. The goal is to predict T-cell epitopes as accurate as possible. For accurate prediction multiple factors need to be taken into account to determine this potential epitope.

The main focus of these predictions is the binding capacity of an epitope to the MHC molecule. However, every step the protein undergoes until the pMHC is formed, is of influence in this

prediction[29]. The epitope prediction strategies can be split into structural based predictions and sequence based predictions.

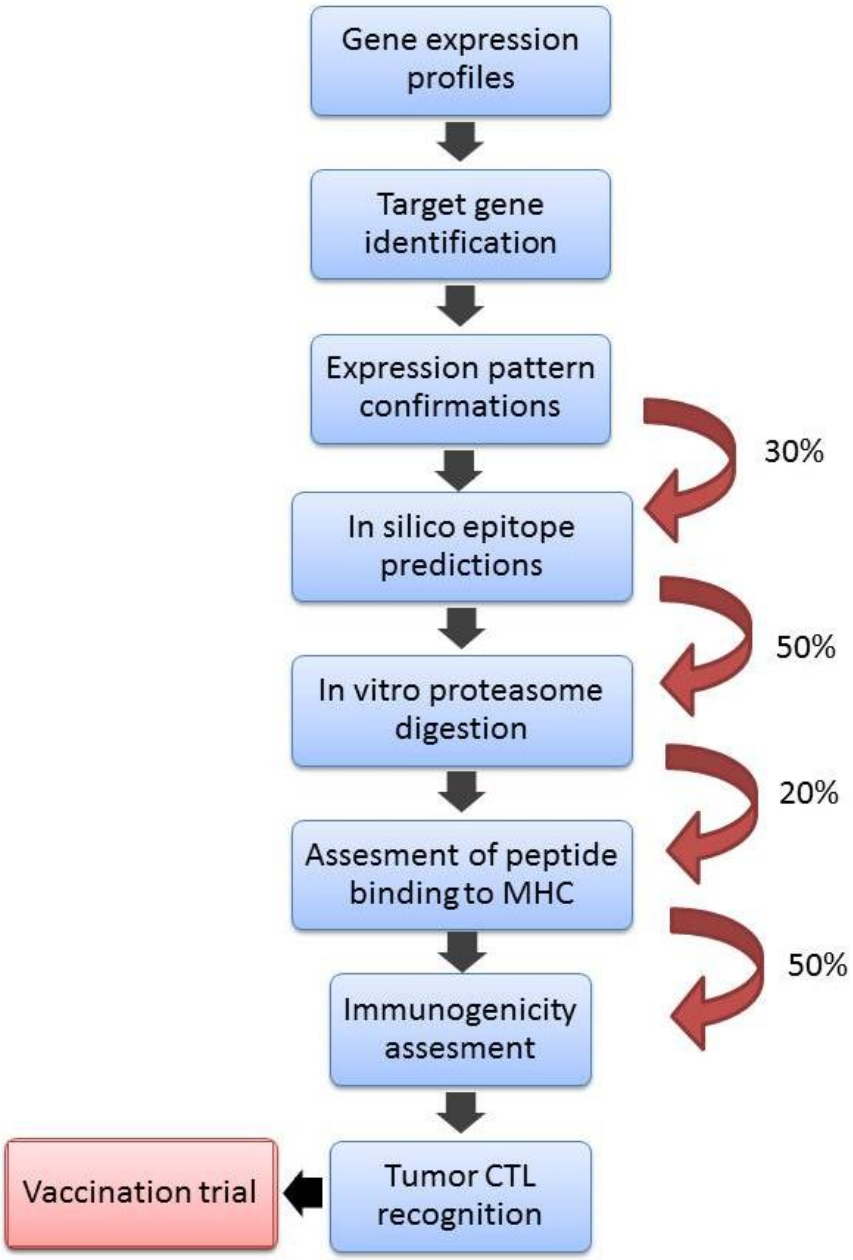


Figure 4. Pipline of reverse immunology epitope identification.
The first step in reverse immunology is to find a gene of interest by looking at gene expression profiles. With the major load of information the best and quickest way to do this is by bioinformatics. However it is also possible to use micro arrays or Westernblot. The next step is to confirming this expected gene expression profile. Of all these peptides confirmed, only 30% remains an option after in silico epitope prediction, since the proteasome does not collide with all peptides. The prediction of proteasome is correct for approximately 50%. The proteins that are capable of proteasome digestion need to be tested for their affinity for a MHC molecule, which is the case for 20% of the peptides. The last group of peptides are then tested for their immunogenicity. A step in which another 50% is lost. The remaining candidates are confirmed with the tumour CTL recognition and if functional can be used in vaccination trials. *(Figure edited from Viatte et al., 2006)*[21]

4.2.1 Sequence based epitope prediction

As mentioned before, the HLA genes are very polymorphic[65]. The differences between HLA molecules are represented in the binding cleft of the molecule. Each allele has its own affinity for different peptides, caused by the difference in sidechains. These HLA sidechains have preferences for different types of peptides to interact with. A peptide with sidechains compatible to those of the HLA ligand can bind the cleft with high affinity. These positions are called the anchor positions[66]. The HLA-A*0201 has been de subject of many HLA studies because it is one of the most common alleles in society. Therefore it is known to have anchor residues at P2, accepting leucine, and at P9, accepting valine, for nonapeptides[21,66].The anchor residue positions is also referred to as a motif.

Epitope predictions based on motifs are one type of sequence-based strategy to predict binding affinity of epitopes. It divides the epitopes into 'binders' and 'non-binders' by comparing the amino acids of possible epitopes to known epitopes with regard to the anchor positions. This strategy already existed in the 1995s when *De Groot et al.* created programs as EpiMer and OptiMer[67]. Motifs are still commonly used, but often in an improved manner. *Rammensee* created the first MHC database: SYFPEITHI because large datasets were needed for comparison and accurate predictions[68]. This is still one of the most used databases for motif predictions.
Improvement of this strategy for example led to the Matrix-Based methods. These methods take every amino acid into account when predicting the peptide-MHC binding capacity, rather than just the anchor residues[69]. One example of a prediction software based on matrices is BIMAS (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD)[70,71].

Another sequence based strategy is based on the Hidden Markov Models (HMM)[72]. Development of the HMM resulted in to the creation of PRED^{tap}, a programme predicting binding affinity of epitopes to TAP[73]. Furthermore programs for HLA peptide binding programs were developed as well, making these HMM suitable for epitope prediction, for example. Moreover this strategy is capable of predicting epitopes for peptides of variable length[69].

One major prerequisite for sequence-based epitope prediction is data to compare with. Therefore extensive databases are required, which is the drawback for this method. A good database should include sequence information of predicted epitopes, but should include validation of these epitopes[71]. Because of its enormous amount of information about peptide and non-peptide epitopes, T-cell assays, MHC binding assays and more Immune Epitope Database (IEDB) is one of the best to use for accurate predictions[74].

4.2.2 Structural based epitope prediction

Another method is the structural based approach. As the name already suggests it predicts binding epitopes by structural information. The structure of the peptide is analysed and the affinity for the peptide-binding cleft is predicted. This method does not need an extensive database, but depends on the 3D structure to evaluate new possible epitopes. However this method is more time-consuming and therefore seems to be less interesting for research. Only limited publications and strategies are developed in this field[75,76].

Predictions made on account of structure can be accomplished by analysing dynamics, or calculating the free energy[77]. Other properties that can influence the affinity are polarity, charge or hydrophilic versus hydrophobic.
One method for this is predicting the ligand docking. This technique relies on the intermolecular interactions, with the goal to accurately predict how well the epitope can bind to the HLA molecule, and in which manner they interact[75]. Another popular approach is 3D threading. In this method a template backbone is the starting point. Calculations are made to predict the folding energy of possible amino acids in all positions, and this is used to fill in the backbone with potential amino acids. Then the interaction energies of all the possible peptide residues are added up to a total. The probability of MHC binding of a peptide is then determined with this information. The process of threading is extensively described by *Altuvia et al.*[77,78]. This approach can be very successful, however since all starts with the template backbone, this backbone can severely influence accuracy[79]. Multiple researchers have taken this approach and tried improving it or extending it by combining it with other methods, which leads to more accurate predictions[80,81].

So *in silico* predictions of epitopes can be performed in a lot of different manners and there is choice from a variety of databases and software. The software of choice should match with the epitope of interest. MHC-I or MHC-II epitopes and type of cancer are two examples of factors that should be taken into account when making this decision. Also accessibility of the site/software is important.

4.3 Confirming predicted epitopes

After making *in silico* predictions for an epitope, this needs to be verified. Since *in silico* predictions are often incorrect, the predicted epitopes need to be tested for their function[82].
In general there are four limiting steps in the process from peptide or protein until epitope presentation by the HLA molecules. The first is processing of the peptide by the proteasome[83]. Then the processed peptide needs to have sufficient affinity for the TAP complex to be transported to the ER. Furthermore, the affinity for the MHC complex is important, and the last step is natural presentation and epitope selection. A peptide chosen for vaccination needs to pass all four steps to be functional. The multiple steps are sometimes included in algorithms already, for example EpiJen[84]. However this is not the case for all algorithms. It is wise to perform *in vitro* assays and check the potential of the peptide for survival of all four steps to prevent disappointing results.

Kessler et al. were the first to introduce improved epitope prediction, as they used 20S proteasomes *in vitro* to test the degradation of predicted epitopes[43]. This technique is used in multiple researches since[85]. The most interesting step in this process is the peptide digestion, which can be analysed with help of Mass Spectrometry (MS)[86]. When a peptide is degraded by the proteasome it needs to be translocated to the ER. For most peptides translocation is not possible through simple diffusion. This transport is then accomplished by TAP, facilitating the peptide translocation to the ER. Therefore it is necessary for the peptide to have sufficient affinity for the TAP complex. The TAP complex is not just needed for transport, it also facilitates peptide trimming of longer peptides and plays an important role in loading the peptide onto the MHC-I complex[87,88]. This should be tested for *in vitro* to make sure presentation of the target peptide is actual possible.

One of the last but most important steps in confirmation of epitopes, is checking immunogenicity of an epitope. This can be performed *in vitro*, but also *in vivo.* In the case of *in vitro* testing CTLs are induced with the target epitope. This starts with the isolation of Peripheral Blood Mononuclear Cells (PBMC). This is commonly done with the Ficoll-Paque method[89]. These are then incubated with the peptides of interest. The goal is for APCs to prime naïve T-cells and create toxic CTLs. The activity of the CTLs is tested. Also performed is a cytoxicity assay, often with the help of ^{51}Cr-release assay[90,91]. However, other techniques for the cytotoxicity assay are available as well[92]. *In vivo* testing is performed in animal models. Because of the use of flow-cytometry analysis of both $CD4^+$ and $CD8^+$ T-cells is possible and easy to perform[93]. Altogether conformation if the epitope can induce CTLs capable of killing tumour cells is important before starting a vaccination trial.

5. Conclusion

Cancer is still a major cause of death worldwide[94] . There is need for the development of novel therapies, preferably less toxic than existing therapies[95]. Peptide anti-tumour peptide vaccination seems to be a good candidate for this. This review has focussed on available epitope identification strategies. Identifying new epitopes can greatly improve the development of new peptide vaccines. With the development of reverse immunology strategies epitope identification can be performed faster and for an increased number of epitopes[21]. However, there is a great number of different strategies available. Also new and improved strategies are always in development[17,20,61].

The best way to perform *in silico* epitope predictions is to take multiple factors of the peptide processing until MHC loading into account. Algorithms that combine these factors, like proteasome degradation and TAP affinity as well as the MHC affinity are therefore the better choice. One good example is EpiJen[84]. Another recommended strategy is the combination of results of two sequence based strategies. Using the epitopes predicted by both HMM and the motif based predictions increases the chance of correct prediction[96]. More important seems to be combining sequence based predictions wit structure based predictions. The combination of the results of these sequence and structure based types of algorithms leads to a 6% increase in accuracy compared to the two methods individually[97]. Moreover, since predictions are based on known epitopes and their sequence and structure, the choice of database for comparison is an important one. IEBD is one of the most extensive databases[74]. Another good database is the MHC binders and non-binders (MHCBN) database, which combines the information of a number of well-known databases with recent literature and web tools like BLAST search[97].

Predicted epitopes still need to be checked for immunogenicity. Both *in vitro* and *in vivo* have their advantages and disadvantages. For potential epitopes the success in human setting can be determined by combining an *in vitro* assay with human PMBCs with an *in vivo* assay performed in transgenic mice[98]. However, it should be carefully considered for each experiment separately if the use of an animal model is of additional value.

When multiple immunogenic epitopes have been identified, it is favourable to administer more epitopes at the same time to generate a broad immune response instead of a single epitope response. However the factor of peptide competition for MHC binding should be taken into account[19]. Interaction between peptides should be researched before vaccination trials. Multiple peptide vaccination could lead to a broad response with positive results if there is no competition between the used peptides[55,99]. If competition is demonstrated the use of multiple vaccinations, administered at different sites of the body, could be a solution to still create a broad response.

When epitopes are identified, the length of the peptide is a major point to take into account. Short peptides are easy and successful in $CD8^+$ CTL induction, however often lead to immunetolerance[47,50]. Even though, the use of short peptides should not be ruled out completely. There are other factors that can have major influence. With the right adjuvants[100], or vaccination intervals[101] immunogenicity

of short peptides can be increased. However, a lot of research would be needed for improvement of these short peptides.

For now vaccination with long peptides containing both $CD8^+$ and $CD4^+$ T-cell epitopes is the more promising option[48]. This method of epitope-linkage has booked great successes[44]. However the drawback in this mechanism is the identification of epitopes with HLA-II affinity. The accuracy of Th cell epitopes predictions is not as good as those for CTL epitopes[102]. The motifs for HLA-II peptide affinity are harder to determine. Moreover, algorithms that include the events in the HLA-II peptide presentation pathway are no quite as developed as those for HLA-I peptide presentation[103]. At the moment one of the best softwares to use for accurate HLA-II predictions is NETMHCIIPAN[104]. With the discovery of more HLA-II epitopes, the epitope-linkage has a bright future in the anti-tumour peptide vaccination development.

One pitfall in epitope selection is ruling out possible epitopes because of 'over selection'. Combinations of multiple strategies increase prediction accuracy, but not too much databases and softwares should be combined, otherwise possibly accurate epitopes can be filtered out. Also, with the right means even low affinity peptides can be enhanced to increase binding stability, and therefore become more immunogenic[105]. Another example is peptides that are filtered out because of low affinity for the TAP complex. In exceptional cases presentation of a peptide can be presented independently of the TAP complex[106]. These are two examples of potential epitopes being filtered out with the use of most algorithms. However these epitopes could come in use, especially in cases where functional epitopes are hard to detect.

With ongoing technological developments, algorithms will be improved like they have been in the past. Together with improvement of confirmation techniques more epitopes will be identified. A good manner of epitope identification with present knowledge has been set out in this paper. With the discovery of more epitopes in different types of cancer, better peptide based anti-tumour vaccination will be developed.

6. References

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